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(54) Title: NOVEL USE

(57) Abstract: The invention provides the use of a) an HIV Tat protein or polynucleotide; or b) an HIV Nef protein or polynucleotide; or c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide (Nef-Tat); and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

NOVEL USE

DESCRIPTION

The present invention relates to novel uses of HIV proteins in medicine and vaccine compositions containing such HIV proteins. In particular, the invention relates to the use of HIV Tat and HIV gp120 proteins in combination. Furthermore, the invention relates to the use of HIV Nef and HIV gp120 proteins in combination.

HIV-1 is the primary cause of the acquired immune deficiency syndrome (AIDS) which is regarded as one of the world's major health problems. Although extensive research throughout the world has been conducted to produce a vaccine, such efforts thus far have not been successful.

The HIV envelope glycoprotein gp120 is the viral protein that is used for attachment to the host cell. This attachment is mediated by the binding to two surface molecules of helper T cells and macrophages, known as CD4 and one of the two chemokine receptors CCR-4 or CXCR-5. The gp120 protein is first expressed as a larger precursor molecule (gp160), which is then cleaved post-translationally to yield gp120 and gp41. The gp120 protein is retained on the surface of the virion by linkage to the gp41 molecule, which is inserted into the viral membrane.

The gp120 protein is the principal target of neutralizing antibodies, but unfortunately the most immunogenic regions of the proteins (V3 loop) are also the most variable parts of the protein. Therefore, the use of gp120 (or its precursor gp160) as a vaccine antigen to elicit neutralizing antibodies is thought to be of limited use for a broadly protective vaccine. The gp120 protein does also contain epitopes that are recognized by cytotoxic T lymphocytes (CTL). These effector cells are able to eliminate virus-infected cells, and therefore constitute a second major antiviral immune mechanism. In contrast to the target regions of neutralizing antibodies some CTL epitopes appear to be relatively conserved among different HIV strains. For this reason gp120 and gp160 are considered to be useful antigenic components in vaccines that aim at eliciting cell-mediated immune responses (particularly CTL).

Non-envelope proteins of HIV-1 have been described and include for example internal structural proteins such as the products of the *gag* and *pol* genes and, other non-structural proteins such as Rev, Nef, Vif and Tat (Greene et al., New England J. Med, **324**, 5, 308 et seq (1991) and Bryant et al. (Ed. Pizzo), Pediatr. Infect. Dis. J., **11**, 5, 390 et seq (1992).

HIV Tat and Nef proteins are early proteins, that is, they are expressed early in infection and in the absence of structural protein.

In a conference presentation (C. David Pauza, Immunization with Tat toxoid attenuates SHIV89.6PD infection in rhesus macaques, 12th Cent Gardes meeting, Marnes-La-Coquette, 26.10.1999), experiments were described in which rhesus macaques were immunised with Tat toxoid alone or in combination with an envelope glycoprotein gp160 vaccine combination (one dose recombinant vaccinia virus and one dose recombinant protein). However, the results observed showed that the presence of the envelope glycoprotein gave no advantage over experiments performed with Tat alone.

However, we have found that a Tat- and/or Nef-containing immunogen (especially a Nef-Tat fusion protein) acts synergistically with gp120 in protecting rhesus monkeys from a pathogenic challenge with chimeric human-simian immunodeficiency virus (SHIV). To date the SHIV infection of rhesus macaques is considered to be the most relevant animal model for human AIDS. Therefore, we have used this preclinical model to evaluate the protective efficacy of vaccines containing a gp120 antigen and a Nef- and Tat-containing antigen either alone or in combination. Analysis of two markers of viral infection and pathogenicity, the percentage of CD4-positive cells in the peripheral blood and the concentration of free SHIV RNA genomes in the plasma of the monkeys, indicated that the two antigens acted in synergy. Immunization with either gp120 or NefTat + SIV Nef alone did not result in any difference compared to immunization with an adjuvant alone. In contrast, the administration of the combination of gp120 and NefTat + SIV Nef, antigens resulted in a marked improvement of the two above-mentioned parameters in all animals of those particular experimental group.

Thus, according to the present invention there is provided a new use of HIV Tat and/or Nef protein together with HIV gp120 in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

As described above, the NefTat protein, the SIV Nef protein and gp120 protein together give an enhanced response over that which is observed when either NefTat + SIV Nef, or gp120 are used alone. This enhanced response, or synergy can be seen in a decrease in viral load as a result of vaccination with these combined proteins. Alternatively, or additionally the enhanced response manifests itself by a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV NefTat, SIV Nef and HIV gp120. The synergistic effect is attributed to the combination of gp120 and Tat, or gp120 and Nef, or gp120 and both Nef and Tat.

The addition of other HIV proteins may further enhance the synergistic effect, which was observed between gp120 and Tat and/or Nef. These other proteins may also act synergistically with individual components of the gp120, Tat and/or Nef-containing vaccine, not requiring the presence of the full original antigen combination. The additional proteins may be regulatory proteins of HIV such as Rev, Vif, Vpu, and Vpr. They may also be structural proteins derived from the HIV *gag* or *pol* genes.

The HIV *gag* gene encodes a precursor protein p55, which can assemble spontaneously into immature virus-like particles (VLPs). The precursor is then proteolytically cleaved into the major structural proteins p24 (capsid) and p18 (matrix), and into several smaller proteins. Both the precursor protein p55 and its major derivatives p24 and p18 may be considered as appropriate vaccine antigens which may further enhance the synergistic effect observed between gp120 and Tat and/or Nef. The precursor p55 and the capsid protein p24 may be used as VLPs or as monomeric proteins.

The HIV Tat protein in the vaccine of the present invention may, optionally be linked to an HIV Nef protein, for example as a fusion protein.

The HIV Tat protein, the HIV Nef protein or the NefTat fusion protein in the present invention may have a C terminal Histidine tail which preferably comprises between 5-10 Histidine residues. The presence of an histidine (or 'His') tail aids purification.

In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 10 and preferably six Histidine residues. These are advantageous in aiding purification. Separate expression, in yeast (Saccharomyces cerevisiae), of Nef (Macreadie I.G. et al., 1993, Yeast 9 (6) 565-573) and Tat (Braddock M et al., 1989, Cell 58 (2) 269-79) has been reported. Nef protein and the Gag proteins p55 and p18 are myristilated. The expression of Nef and Tat separately in a Pichia expression system (Nef-His and Tat-His constructs), and the expression of a fusion construct Nef-Tat-His have been described previously in WO99/16884.

The DNA and amino acid sequences of representative Nef-His (Seq. ID. No.s 8 and 9), Tat-His (Seq. ID. No.s 10 and 11)and of Nef-Tat-His fusion proteins (Seq. ID. No.s 12 and 13) are set forth in Figure 1.

The HIV proteins of the present invention may be used in their native conformation, or more preferably, may be modified for vaccine use. These modifications may either be required for technical reasons relating to the method of purification, or they may be used to biologically inactivate one or several functional properties of the Tat or Nef protein. Thus the invention encompasses derivatives of HIV proteins which may be, for example mutated proteins. The term 'mutated' is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method.

For example, a mutant Tat protein may be mutated so that it is biologically inactive whilst still maintaining its immunogenic epitopes. One possible mutated tat gene, constructed by D.Clements (Tulane University), (originating from BH10 molecular clone) bears mutations in the active site region (Lys41→Ala)and in RGD motif (Arg78→Lys and Asp80→Glu) (Virology 235: 48-64, 1997).

A mutated Tat is illustrated in Figure 1 (Seq. ID. No.s 22 and 23) as is a Nef-Tat Mutant-His (Seq. ID. No.s 24 and 25).

The HIV Tat or Nef proteins in the vaccine of the present invention may be modified by chemical methods during the purification process to render the proteins stable and monomeric. One method to prevent oxidative aggregation of a protein such as Tat or Nef is the use of chemical modifications of the protein's thiol groups. In a first step the disulphide bridges are reduced by treatment with a reducing agent such as DTT, beta-mercaptoethanol, or gluthatione. In a second step the resulting thiols are blocked by reaction with an alkylating agent (for example, the protein can be carboxyamidated/carbamidomethylated using iodoacetamide). Such chemical modification does not modify functional properties of Tat or Nef as assessed by cell binding assays and inhibition of lymphoproliferation of human peripheral blood mononuclear cells.

The HIV Tat protein and HIV gp120 proteins can be purified by the methods outlined in the attached examples.

The vaccine of the present invention will contain an immunoprotective or immunotherapeutic quantity of the Tat and/or Nef or NefTat and gp120 antigens and may be prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in the vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 μg of each

protein, preferably 2-200 μg, most preferably 4-40 μg of Tat or Nef or NefTat and preferably 1-150 μg, most preferably 2-25 μg of gp120. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. One particular example of a vaccine dose will comprise 20 μg of NefTat and 5 or 20 μg of gp120. Following an initial vaccination, subjects may receive a boost in about 4 weeks, and a subsequent second booster immunisation.

The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Adjuvants are described in general in Vaccine Design – the Subunit and Adjuvant Approach, edited by Powell and Newman, Plenum Press, New York, 1995.

Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

In the formulation of the invention it is preferred that the adjuvant composition induces a preferential Th1 response. However it will be understood that other responses, including other humoral responses, are not excluded.

An immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. The resultant immune response may be broadly distinguished into two extreme catagories, being humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral response).

Extreme Th1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice Th1-type responses are often characterised by the generation of

antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. Th2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

It can be considered that the driving force behind the development of these two types of immune responses are cytokines, a number of identified protein messengers which serve to help the cells of the immune system and steer the eventual immune response to either a Th1 or Th2 response. Thus high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2- type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor-β(TNF-β).

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2 - type cytokine responses. Traditionally the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection includes direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*, and induces antigen specific immunoglobulin responses associated with Th1-type isotype.

Preferred Th1-type immunostimulants which may be formulated to produce adjuvants suitable for use in the present invention include and are not restricted to the following.

Monophosphoryl lipid A, in particular 3-de-O-acylated monophosphoryl lipid A (3D-MPL), is a preferred Th1-type immunostimulant for use in the invention. 3D-MPL is a well known adjuvant manufactured by Ribi Immunochem, Montana. Chemically it is often supplied as a mixture of 3-de-O-acylated monophosphoryl lipid A with either 4, 5, or 6 acylated chains. It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1). A preferred form of 3D-MPL is in the form of a particulate formulation having a small particle size less than 0.2μm in diameter, and its method of manufacture is disclosed in EP 0 689 454.

Saponins are also preferred Th1 immunostimulants in accordance with the invention. Saponins are well known adjuvants and are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Also described in these references is the use of QS7 (a non-haemolytic fraction of Quil-A) which acts as a potent adjuvant for systemic vaccines. Use of QS21 is further described in Kensil *et al.* (1991. J.

Immunology vol 146, 431-437). Combinations of QS21 and polysorbate or cyclodextrin are also known (WO 99/10008). Particulate adjuvant systems comprising fractions of QuilA, such as QS21 and QS7 are described in WO 96/33739 and WO 96/11711.

Another preferred immunostimulant is an immunostimulatory oligonucleotide containing unmethylated CpG dinucleotides ("CpG"). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA. CpG is known in the art as being an adjuvant when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). Historically, it was observed that the DNA fraction of BCG could exert an anti-tumour effect. In further studies, synthetic oligonucleotides derived from BCG gene sequences were shown to be capable of inducing immunostimulatory effects (both in vitro and in vivo). The authors of these studies concluded that certain palindromic sequences, including a central CG motif, carried this activity. The central role of the CG motif in immunostimulation was later elucidated in a publication by Krieg, Nature 374, p546 1995. Detailed analysis has shown that the CG motif has to be in a certain sequence context, and that such sequences are common in bacterial DNA but are rare in vertebrate DNA. The immunostimulatory sequence is often: Purine, Purine, C, G, pyrimidine, pyrimidine; wherein the CG motif is not methylated, but other unmethylated CpG sequences are known to be immunostimulatory and may be used in the present invention.

In certain combinations of the six nucleotides a palindromic sequence is present. Several of these motifs, either as repeats of one motif or a combination of different motifs, can be present in the same oligonucleotide. The presence of one or more of these immunostimulatory sequences containing oligonucleotides can activate various immune subsets, including natural killer cells (which produce interferon γ and have cytolytic activity) and macrophages (Wooldrige et al Vol 89 (no. 8), 1977). Other unmethylated CpG containing sequences not having this consensus sequence have also now been shown to be immunomodulatory.

CpG when formulated into vaccines, is generally administered in free solution together with free antigen (WO 96/02555; McCluskie and Davis, *supra*) or covalently conjugated to an antigen (WO 98/16247), or formulated with a carrier such as aluminium hydroxide ((Hepatitis surface antigen) Davis *et al. supra* ; Brazolot-Millan *et al., Proc.Natl.Acad.Sci.*, USA, 1998, 95(26), 15553-8).

Such immunostimulants as described above may be formulated together with carriers, such as for example liposomes, oil in water emulsions, and or metallic salts, including aluminium salts (such as aluminium hydroxide). For example, 3D-MPL may be formulated with aluminium hydroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210); QS21 may be advantageously formulated with cholesterol containing liposomes (WO 96/33739), oil in water emulsions (WO 95/17210) or alum (WO 98/15287); CpG may be formulated with alum (Davis *et al. supra* ; Brazolot-Millan *supra*) or with other cationic carriers.

Combinations of immunostimulants are also preferred, in particular a combination of a monophosphoryl lipid A and a saponin derivative (WO 94/00153; WO 95/17210; WO 96/33739; WO 98/56414; WO 99/12565; WO 99/11241), more particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153. Alternatively, a combination of CpG plus a saponin such as QS21 also forms a potent adjuvant for use in the present invention.

Thus, suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3D-MPL, together with an aluminium salt.
An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched in cholesterol containing liposomes (DQ) as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is another preferred formulation for use in the invention.

Another preferred formulation comprises a CpG oligonucleotide alone or together with an aluminium salt.

In another aspect of the invention, the vaccine may contain DNA encoding one or more of the Tat, Nef and gp120 polypeptides, such that the polypeptide is generated *in situ*. The DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems such as plasmid DNA, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, Crit. Rev. Therap. Drug Carrier Systems 15:143-198, 1998 and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). When the expression system is a recombinant live microorganism, such as a virus or bacterium, the gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox, modified poxviruses e.g. Modified Virus Ankara (MVA)), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), flaviviruses (yellow fever virus, Dengue virus, Japanese encephalitis virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella , Shigella, Neisseria, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

Thus, the Nef, Tat and gp120 components of a preferred vaccine according to the invention may be provided in the form of polynucleotides encoding the desired proteins.

Furthermore, immunisations according to the invention may be performed with a combination of protein and DNA-based formulations. Prime-boost immunisations are considered to be effective in inducing broad immune responses. Adjuvanted protein vaccines induce mainly antibodies and T helper immune responses, while delivery of DNA as a plasmid or a live vector induces strong cytotoxic T lymphocyte (CTL)

responses. Thus, the combination of protein and DNA vaccination will provide for a wide variety of immune responses. This is particularly relevant in the context of HIV, since both neutralising antibodies and CTL are thought to be important for the immune defence against HIV.

In accordance with the invention a schedule for vaccination with gp120, Nef and Tat, alone or in combination, may comprise the sequential ("prime-boost") or simultaneous administration of protein antigens and DNA encoding the above-mentioned proteins. The DNA may be delivered as plasmid DNA or in the form of a recombinant live vector, e.g. a poxvirus vector or any other suitable live vector such as those described herein. Protein antigens may be injected once or several times followed by one or more DNA administrations, or DNA may be used first for one or more administrations followed by one or more protein immunisations.

A particular example of prime-boost immunisation according to the invention involves priming with DNA in the form of a recombinant live vector such as a modified poxvirus vector, for example Modified Virus Ankara (MVA) or a alphavirus, for example Venezuelian Equine Encephalitis Virus followed by boosting with a protein, preferably an adjuvanted protein.

Thus the invention further provides a pharmaceutical kit comprising:

a) a composition comprising one or more of gp120, Nef and Tat proteins together with a pharmaceutically acceptable excipient; and

b) a composition comprising one or more of gp120, Nef and Tat-encoding polynucleotides together with a pharmaceutically acceptable excipient;

with the proviso that at least one of (a) or (b) comprises gp120 with Nef and/or Tat and/or Nef-Tat.

Compositions a) and b) may be administered separately, in any order, or together. Preferably a) comprises all three of gp120, Nef and Tat proteins. Preferably b) comprises all three of gp120, Nef and Tat DNA. Most preferably the Nef and Tat are in the form of a NefTat fusion protein.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine formulation as herein described, wherein the method comprises admixing

a combination of proteins according to the invention. The protein composition may be mixed with a suitable adjuvant and, optionally, a carrier.

Particularly preferred adjuvant and/or carrier combinations for use in the formulations according to the invention are as follows:

i) 3D-MPL + QS21 in DQ

ii) Alum + 3D-MPL

iii) Alum + QS21 in DQ + 3D-MPL

iv) Alum + CpG

v) 3D-MPL + QS21 in DQ + oil in water emulsion

vi) CpG

The invention is illustrated in the accompanying examples and Figures:

EXAMPLES

General

The Nef gene from the Bru/Lai isolate (Cell 40: 9-17, 1985) was selected for the constructs of these experiments since this gene is among those that are most closely related to the consensus Nef .

The starting material for the Bru/Lai Nef gene was a 1170bp DNA fragment cloned on the mammalian expression vector pcDNA3 (pcDNA3/Nef).

The Tat gene originates from the BH10 molecular clone. This gene was received as an HTLV III cDNA clone named pCV1 and described in Science, 229, p69-73, 1985.

The expression of the Nef and Tat genes could be in Pichia or any other host.

Example 1. EXPRESSION OF HIV-1 *nef* AND *tat* SEQUENCES IN PICHIA PASTORIS.

Nef protein, Tat protein and the fusion Nef -Tat were expressed in the methylotrophic yeast *Pichia pastoris* under the control of the inducible alcohol oxidase (AOX1) promoter.

To express these HIV-1 genes a modified version of the integrative vector PHIL-D2 (INVITROGEN) was used. This vector was modified in such a way that expression of heterologous protein starts immediately after the native ATG codon of the AOX1 gene and will produce recombinant protein with a tail of one glycine and six histidines residues . This PHIL-D2-MOD vector was constructed by cloning an oligonucleotide linker between the adjacent AsuII and EcoRI sites of PHIL-D2 vector (see Figure 2). In addition to the His tail, this linker carries NcoI, SpeI and XbaI restriction sites between which *nef*, *tat* and *nef-tat* fusion were inserted.

1.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14597 (encoding Nef-His protein), pRIT14598 (encoding Tat-His protein) and pRIT14599 (encoding fusion Nef-Tat-His).

The *nef* gene was amplified by PCR from the pcDNA3/Nef plasmid with primers 01 and 02.

NcoI

PRIMER 01 (Seq ID NO 1): 5'ATCGTCC**ATG.**GGT.GGC.AAG.TGG.T 3'

SpeI

PRIMER 02 (Seq ID NO 2): 5' CGGCTACTAGTGCAGTTCTTGAA 3'

The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14597 (see Figure 2).

The *tat* gene was amplified by PCR from a derivative of the pCV1 plasmid with primers 05 and 04:

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

NcoI

PRIMER 05 (Seq ID NO 5): 5'ATCGTCC**ATG**GAGCCAGTAGATC 3'

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained

and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14598.

To construct pRIT14599, a 910bp DNA fragment corresponding to the *nef-tat*-His coding sequence was ligated between the EcoRI blunted(T4 polymerase) and NcoI sites of the PHIL-D2-MOD vector. The *nef-tat*-His coding fragment was obtained by XbaI blunted(T4 polymerase) and NcoI digestions of pRIT14596.

1.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strains expressing Nef-His, Tat-His and the fusion Nef-Tat-His, strain GS115 was transformed with linear NotI fragments carrying the respective expression cassettes plus the HIS4 gene to complement his4 in the host genome.Transformation of GS115 with NotI-linear fragments favors recombination at the AOXI locus.

Multicopy integrant clones were selected by quantitative dot blot analysis and the type of integration, insertion (Mut$^+$phenotype) or transplacement (Mut^{s}phenotype), was determined.

From each transformation, one transformant showing a high production level for the recombinant protein was selected :

Strain Y1738 (Mut$^+$ phenotype) producing the recombinant Nef-His protein, a myristylated 215 amino acids protein which is composed of:

°Myristic acid

°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector

°205 a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)

°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector.

°One glycine and six histidines.

Strain Y1739 (Mut^+ phenotype) producing the Tat-His protein, a 95 amino acid protein which is composed of:

°A methionine created by the use of NcoI cloning site
°85 a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)

°A threonine and a serine introduced by cloning procedure
°One glycine and six histidines

Strain Y1737(Mut^s phenotype) producing the recombinant Nef-Tat-His fusion protein, a myristylated 302 amino acids protein which is composed of:

°Myristic acid
°A methionine, created by the use of NcoI cloning site
°205a.a. of Nef protein(starting at a.a.2 and extending to a.a.206)
°A threonine and a serine created by the cloning procedure
°85a.a. of the Tat protein(starting at a.a.2 and extending to a.a.86)
°A threonine and a serine introduced by the cloning procedure
°One glycine and six histidines

Example 2. **<u>EXPRESSION OF HIV-1 Tat-MUTANT IN PICHIA PASTORIS</u>**

A mutant recombinant Tat protein has also been expressed. The mutant Tat protein must be **biologically inactive** while **maintaining** its **immunogenic epitopes.**

A double mutant *tat* gene, constructed by D.Clements (Tulane University) was selected for these constructs.

This *tat* gene (originates from BH10 molecular clone) bears **mutations** in the **active site region (Lys41→Ala)**and in **RGD motif** (**Arg78→Lys and Asp80→Glu**) (Virology 235: 48-64, 1997).

The mutant *tat* gene was received as a cDNA fragment subcloned between the EcoRI and HindIII sites within a CMV expression plasmid (pCMVLys41/KGE)

2.1 CONSTRUCTION OF THE INTEGRATIVE VECTORS pRIT14912(encoding Tat mutant-His protein) and pRIT14913(encoding fusion Nef-Tat mutant-His).

The *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 05 and 04 (see section 1.1construction of pRIT14598)

An NcoI restriction site was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end with primer 04. The PCR fragment obtained and the PHIL-D2-MOD vector were both restricted by NcoI and SpeI, purified on agarose gel and ligated to create the integrative plasmid pRIT14912

To construct pRIT14913, the *tat* mutant gene was amplified by PCR from the pCMVLys41/KGE plasmid with primers 03 and 04.

SpeI

PRIMER 03 (Seq ID NO 3): 5' ATCGTACTAGT.GAG.CCA.GTA.GAT.C 3'

SpeI

PRIMER 04 (Seq ID NO 4): 5' CGGCTACTAGTTTCCTTCGGGCCT 3'

The PCR fragment obtained and the plasmid pRIT14597 (expressing Nef-His protein) were both digested by SpeI restriction enzyme, purified on agarose gel and ligated to create the integrative plasmid pRIT14913

2.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115.

Pichia pastoris strains expressing Tat mutant-His protein and the fusion Nef-Tat mutant-His were obtained, by applying integration and recombinant strain selection strategies previously described in section 1.2 .

Two recombinant strains producing Tat mutant-His protein ,a 95 amino-acids protein, were selected: Y1775 (Mut^+ phenotype) and Y1776(Mut^s phenotype).

One recombinant strain expressing Nef-Tat mutant-His fusion protein, a 302 amino-acids protein was selected: Y1774(Mut^+ phenotype).

Example 3: FERMENTATION OF PICHIA PASTORIS PRODUCING RECOMBINANT TAT-HIS.

A typical process is described in the table hereafter.

Fermentation includes a growth phase (feeding with a glycerol-based medium according to an appropriate curve) leading to a high cell density culture and an induction phase (feeding with a methanol and a salts/micro-elements solution). During fermentation the growth is followed by taking samples and measuring their absorbance at 620 nm. During the induction phase methanol was added via a pump and its concentration monitored by Gas chromatography (on culture samples) and by on-line gas analysis with a Mass spectrometer. After fermentation the cells were recovered by centrifugation at 5020g during 30' at 2-8°C and the cell paste stored at –20°C. For further work cell paste was thawed, resuspended at an OD (at 620 nm) of 150 in a buffer (Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM) and disrupted by 4 passages in a DynoMill (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).

For evaluation of the expression samples were removed during the induction, disrupted and analyzed by SDS-Page or Western blot. On Coomassie blue stained SDS-gels the recombinant Tat-his was clearly identified as an intense band presenting a maximal intensity after around 72-96H induction.

Thawing of a Working .seed vial	
↓	
Solid preculture 30°C, 14-16H	Synthetic medium: YNB + glucose + agar
↓	
Liquid preculture in two 2L erlenmeyer 30°C, 200 rpm	Synthetic medium: 2 x 400 ml YNB + glycerol Stop when OD > 1 (at 620 nm)
↓	
Inoculation of a 20L fermentor	5L initial medium (FSC006AA) 3 ml antifoam SAG471 (from Witco) Set-points: Temperature : 30°C Overpressure: 0.3 barg Air flow: 20 Nl/min Dissolved O2: regulated > 40% pH : regulated at 5 by NH_4OH
↓	
Fed-batch fermentation: growth phase Duration around 40H,	Feeding with glycerol-based medium FFB005AA Final OD between 200-500 OD (620 nm)
Fed-batch fermentation: induction phase Duration: up to 97H.	Feeding with methanol and with a salt/micro-elements solution (FSE021AB).
↓	
Centrifugation	5020g /30 min / 2-8°C
↓	
Recover cell paste and store at –20°C	
↓	
Thaw cells and resuspend at OD150 (620 nm) in buffer	Buffer: Na2HPO4 pH7 50 mM, PMSF 5%, Isopropanol 4 mM
↓	
Cell disruption in Dyno-mill 4 passages	Dyno-mill: (room 0.6L, 3000 rpm, 6L/H, beads diameter of 0.40-0.70 mm).
↓	
Transfer for extraction/purification	

Media used for fermentation:

Solid preculture: (YNB + glucose + agar)

Glucose:	10 g/l	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	0.000064 g/l
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	0.000016 g/l
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l	Agar	18 g/l

Liquid preculture ,(YNB + glycerol)

Glycerol:	2% (v/v)	Na2MoO4.2H2O:	0.0002 g/l	Acide folique:	0.000064 g/l
KH2PO4:	1 g/l	MnSO4.H2O:	0.0004 g/l	Inositol:	0.064 g/l
MgSO4.7H2O:	0.5 g/l	H3BO3:	0.0005 g/l	Pyridoxine:	0.008 g/l
CaCl2.2H2O:	0.1 g/l	KI:	0.0001 g/l	Thiamine:	0.008 g/l
NaCl:	0.1 g/l	CoCl2.6H2O:	0.00009 g/l	Niacine:	0.000032 g/l
FeCl3.6H2O:	0.0002 g/l	Riboflavine:	0.000016 g/l	Panthoténate Ca:	0.008 g/l
CuSO4.5H2O:	0.00004 g/l	Biotine:	0.000064 g/l	Para-aminobenzoic acid:	0.000016 g/l
ZnSO4.7H2O:	0.0004 g/l	(NH4)2SO4:	5 g/l		

Initial fermentor charge: (FSC006AA)

(NH4)$_2$SO4:	6.4 g/l		
KH2PO4:	9 g/l	Na2MoO4.2H2O:	2.04 mg/l
MgSO4.7H2O:	4.7 g/l	MnSO4.H2O:	4.08 mg/l
CaCl2.2H2O:	0.94 g/l	H3BO3:	5.1 mg/l
FeCl3.6H2O:	10 mg/l	KI:	1.022 mg/l
HCl:	1.67 ml/l	CoCl2.6H2O:	0.91mg/l
CuSO4.5H2O:	0.408 mg/l	NaCl:	0.06 g/l
ZnSO4.7H2O:	4.08 mg/l	Biotine:	0.534 mg/l

Feeding solution used for growth phase (FFB005AA)

Glycérol:	38.7 % v/v	Na2MoO4.2H2O:	5.7 mg/l
MgSO4.7H2O:	13 g/l	CuSO4.5H2O:	1.13 mg/l
CaCl2.2H2O:	2.6 g/l	CoCl2.6H2O:	2.5 mg/l
FeCl3.6H2O:	27.8mg/l	H3BO3:	14.2 mg/l
ZnSO4.7H2O	11.3 mg/l	Biotine:	1.5 mg/l
MnSO4.H2O:	11.3 mg/l	KI:	2.84mg/l
KH2PO4:	24.93 g/l	NaCl:	0.167 g/l

Feeding solution of salts and micro-elements used during induction (FSE021AB):

KH2PO4:	45 g/l	Na2MoO4.2H2O:	10.2 mg/l
MgSO4.7H2O:	23.5 g/l	MnSO4.H2O:	20.4 mg/l
CaCl2.2H2O:	4.70 g/l	H3BO3:	25.5 mg/l
NaCl:	0.3 g/l	KI:	5.11 mg/l
HCl:	8.3 ml/l	CoCl2.6H2O:	4.55mg/l
CuSO4.5H2O:	2.04 mg/l	FeCl3.6H2O:	50.0 mg/l
ZnSO4.7H2O:	20.4 mg/l	Biotine:	2.70 mg/l

Example 4: PURIFICATION OF Nef-Tat-His FUSION PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 146g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 55. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

146g of Pichia pastoris cells

↓

Homogenization — Buffer: 2L 50 mM PO_4 pH 7.0

final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation — JA10 rotor / 9500 rpm/ 30 min / room temperature

↓

Dyno-mill Pellet

↓

Wash (1h - 4°C) — Buffer: +2L 10 mM PO_4 pH 7.5 - 150mM - NaCl 0,5% empigen

↓

Centrifugation — JA10 rotor / 9500 rpm/ 30 min / room temperature

↓

Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 660ml 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Reduction (4H – room temperature - in the dark)	+ 0,2M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
carbamidomethylation (1/2 h – room temperature - in the dark)	+ 0,25M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 0,5M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea 3) 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 25 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea - 0,5M Imidazol

↓	
Dilution	Down to an ionic strength of 18 mS/cm^2 Dilution buffer: 10 mM PO_4 pH 7.5 - 6M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6.0M Urea Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 - 250mM NaCl - 6M Urea Elution buffer: 10 mM Borate pH 9.0 - 2M NaCl - 6M Urea
↓	
Concentration	up to 5 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex200 XK 16/60 (Pharmacia - 120 ml of resin)	Elution buffer: 10 mM PO_4 pH 7.5 - 150mM NaCl - 6M Urea 5 ml of sample / injection → 5 injections
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 - 150mM NaCl - 0,5M Arginin*
↓	
Sterile filtration	Millex GV 0,22µm

* ratio: 0,5M Arginin for a protein concentration of 1600µg/ml.

Purity

The level of purity as estimated by SDS-PAGE is shown in Figure 3 by Daiichi Silver Staining and in Figure 4 by Coomassie blue G250.

After Superdex200 step:	> 95%
After dialysis and sterile filtration steps:	> 95%

Recovery

51mg of Nef-Tat-his protein are purified from 146g of recombinant Pichia pastoris cells (= 2L of Dyno-mill homogenate OD 55)

Example 5: PURIFICATION OF OXIDIZED NEF-TAT-HIS FUSION PROTEIN IN PICHIA PASTORIS

The purification scheme has been developed from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps , Nef-Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

73 g of Pichia pastoris cells

↓

Homogenization — Buffer: 1L 50 mM PO_4 pH 7.0 – Pefabloc 5 mM

final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash **(2h - 4°C)**	Buffer: +1L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 0,5% Empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation **(O/N - 4°C)**	Buffer: + 330ml 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 15 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 18 mS/cm² Dilution buffer: 10 mM PO_4 pH 7.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF **(Pharmacia - 7 ml of resin)**	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 250 mM NaCl – 6 M Urea

	Elution buffer: 10 mM Borate pH 9.0 – 2 M NaCl – 6 M Urea
↓	
Concentration	up to 0,8 mg/ml 10kDa Omega membrane(Filtron)
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl – 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22µm

➔ Level of purity estimated by SDS-PAGE is shown in Figure 6 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

2,8 mg of oxidized Nef-Tat-his protein are purified from 73 g of recombinant Pichia pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 50.

Example 6: PURIFICATION OF REDUCED TAT-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 2L Dyno-mill homogenate OD 66. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

160 g of Pichia pastoris cells	
↓	
Homogenization	Buffer: +2 L 50 mM PO_4 pH 7.0 – 4 mM PMSF final OD:66
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (1h - 4°C)	Buffer: +2 L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 1% Empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 660 ml 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Reduction (4H - room temperature - in the dark)	+ 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
carbamidomethylation (1/2 h - room temperature - in the dark)	+ 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 60 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM

	NaCl - 6M Urea - 35 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 30 ml of resin)	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: Equilibration buffer Elution buffer: 20 mM Borate pH 8.5 – 400 mM NaCl - 6.0 M Urea
↓	
Concentration	up to 1,5 mg/ml 10kDa Omega membrane(Filtron)
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl - 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22 µm

➔ Level of purity estimated by SDS-PAGE as shown in Figure 7(Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

48 mg of reduced Tat-his protein are purified from 160 g of recombinant Pichia pastoris cells (wet weight) or 2 L of Dyno-mill homogenate OD 66.

Example 7: Purification of oxidized Tat-his protein (Pichia Pastoris)

The purification scheme has been developed from 74 g of recombinant Pichia pastoris cells (wet weight) or 1L Dyno-mill homogenate OD60. The chromatographic steps are performed at room temperature. Between steps, Tat positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

74 g of Pichia pastoris cells	
↓	
Homogenization	<u>Buffer</u>: +1 L 50 mM PO_4 pH 7.0 – 5 mM Pefabloc final OD:60
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Wash (1h - 4°C)	<u>Buffer</u>:+1 L 10 mM PO_4 pH 7.5 – 150 mM NaCl - 1% Empigen
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Pellet	
↓	
Solubilisation (O/N - 4°C)	<u>Buffer</u>: + 330 ml 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	

Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 30 ml of resin)	Equilibration buffer: 10 mM PO_4 pH 7.5 –150 mM NaCl - 4.0 M GuHCl Washing buffer: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 35 mM Imidazol Elution buffer: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Dilution	Down to an ionic strength of 12 mS/cm Dilution buffer: 20 mM Borate pH 8.5 – 6 M Urea
↓	
Cation exchange chromatography on SP Sepharose FF (Pharmacia - 15 ml of resin)	Equilibration buffer: 20 mM Borate pH 8.5 - 150 mM NaCl - 6.0 M Urea Washing buffer: 1) Equilibration buffer 2) 20 mM Borate pH 8.5 - 400 mM NaCl - 6.0 M Urea Elution buffer: 20 mM Piperazine pH 11.0 – 2 M NaCl – 6 M Urea
↓	
Concentration	up to 1,5 mg/ml 10 kDa Omega membrane(Filtron)
↓	
Dialysis (O/N - 4°C)	Buffer: 10 mM PO_4 pH 6.8 – 150 mM NaCl - 0,5 M Arginin
↓	
Sterile filtration	Millex GV 0,22 μm

➔ Level of purity estimated by SDS-PAGE as shown in Figure 8 (Daiichi Silver Staining, Coomassie blue G250, Western blotting):

After dialysis and sterile filtration steps: > 95%

➔ Recovery (evaluated by a colorimetric protein assay: DOC TCA BCA)

19 mg of oxidized Ta:-his protein are purified from 74 g of recombinant Pichia pastoris cells (wet weight) or 1 L of Dyno-mill homogenate OD 60.

Example 8: PURIFICATION OF SIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L Dyno-mill homogenate OD 100. The chromatographic steps are performed at room temperature. Between steps , Nef positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

340 g of Pichia pastoris cells	
↓	
Homogenization	Buffer: 4L 50 mM PO_4 pH 7.0 – PMSF 4 mM final OD:100
↓	
Dyno-mill disruption (4 passes)	
↓	
Centrifugation	JA10 rotor / 9500 rpm/ 60 min / room temperature
↓	
Dyno-mill Pellet	
↓	
Solubilisation (O/N - 4°C)	Buffer: + 2,6 L 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl
↓	
Centrifugation	JA10 rotor / 9500 rpm / 30 min / room temperature
↓	
Reduction (4H - room temperature - in the dark)	+ 0,2 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with

	1 M NaOH solution) before incubation
↓	
Carbamidomethylation **(1/2 h - room temperature - in the dark)**	+ 0,25 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 40 ml of resin)	<u>Equilibration buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl <u>Washing buffer</u>: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol <u>Elution buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex 200 **(Pharmacia - 120 ml of resin)**	<u>Elution buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea
↓	
Concentration	up to 1,5 mg/ml 10kDa Omega membrane(Filtron)
↓	
Dialysis **(O/N - 4°C)**	<u>Buffer</u>: 10 mM PO_4 pH 6.8 – 150 mM NaCl – Empigen 0,3%
↓	
Sterile filtration	Millex GV 0,22µm

➔ <u>Level of purity estimated by SDS-PAGE as shown in Figure 9 (Daiichi Silver Staining, Coomassie blue G250, Western blotting)</u>:

After dialysis and sterile filtration steps: > 95%

➔ <u>Recovery</u> (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of SIV reduced Nef -his protein are purified from 340 g of recombinant Pichia pastoris cells (wet weight) or 4 L of Dyno-mill homogenate OD 100.

Example 9: PURIFICATION OF HIV REDUCED NEF-HIS PROTEIN (PICHIA PASTORIS)

The purification scheme has been developed from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L Dyno-mill homogenate OD 50. The chromatographic steps are performed at room temperature. Between steps , Nef positive fractions are kept overnight in the cold room (+4°C) ; for longer time, samples are frozen at -20°C.

160 g of Pichia pastoris cells

↓

Homogenization — Buffer: 3 L 50 mM PO_4 pH 7.0 – Pefabloc 5 mM final OD:50

↓

Dyno-mill disruption (4 passes)

↓

Freezing/Thawing

↓

Centrifugation — JA10 rotor / 9500 rpm/ 60 min / room temperature

↓

Dyno-mill Pellet

↓

Solubilisation (O/N - 4°C) — Buffer: + 1 L 10 mM PO_4 pH 7.5 - 150mM NaCl - 4.0M GuHCl

↓

Centrifugation — JA10 rotor / 9500 rpm / 60 min / room temperature

↓

Reduction **(3 H - room temperature - in the dark)**	+ 0,1 M 2-mercaptoethanesulfonic acid, sodium salt (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Carbamidomethylation **(1/2 h - room temperature - in the dark)**	+ 0,15 M Iodoacetamide (powder addition) / pH adjusted to 7.5 (with 1 M NaOH solution) before incubation
↓	
Immobilized metal ion affinity chromatography on Ni^{++}-NTA-Agarose (Qiagen - 10 ml of resin)	<u>Equilibration buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM NaCl - 4.0 M GuHCl <u>Washing buffer</u>: 1) Equilibration buffer 2) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea 3) 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea - 25 mM Imidazol <u>Elution buffer</u>: 10 mM Citrate pH 6.0 - 150 mM NaCl – 6 M Urea - 0,5 M Imidazol
↓	
Concentration	up to 3 mg/ml 10kDa Omega membrane(Filtron)
↓	
Gel filtration chromatography on Superdex 200 **(Pharmacia - 120 ml of resin)**	<u>Elution buffer</u>: 10 mM PO_4 pH 7.5 – 150 mM NaCl – 6 M Urea
↓	
Dialysis **(O/N - 4°C)**	<u>Buffer</u>: 10 mM PO_4 pH 6.8 – 150 mM NaCl – 0,5M Arginin
↓	
Sterile filtration	Millex GV 0,22µm

➔ <u>Level of purity estimated by SDS-PAGE as shown in Figure 10 (Daiichi Silver Staining, Coomassie blue G250, Western blotting)</u>:

After dialysis and sterile filtration steps: > 95%

➔ <u>Recovery</u> (evaluated by a colorimetric protein assay: DOC TCA BCA)

20 mg of HIV reduced Nef -his protein are purified from 160 g of recombinant Pichia pastoris cells (wet weight) or 3 L of Dyno-mill homogenate OD 50.

Example 10: EXPRESSION OF SIV *nef* SEQUENCE IN *PICHIA PASTORIS*

In order to evaluate Nef and Tat antigens in the pathogenic SHIV challenge model, we have expressed the Nef protein of simian immunodeficiency virus (SIV) of macaques, SIVmac239 (Aids Research and Human Retroviruses, 6:1221-1231,1990).
In the Nef coding region , SIV mac 239 has an in-frame stop codon after 92aa predicting a truncated product of only 10kD. The remainder of the Nef reading frame is open and would be predicted to encode a protein of 263aa (30kD) in its fully open form.

Our starting material for SIVmac239 *nef* gene was a DNA fragment corresponding to the complete coding sequence, cloned on the LX5N plasmid (received from Dr R.C. Desrosiers, Southborough,MA,USA) .
This SIV *nef* gene is mutated at the premature stop codon (nucleotide G at position 9353 replaces the original T nucleotide) in order to express the full-length SIVmac239 Nef protein.

To express this SIV *nef* gene in <u>*Pichia pastoris*</u>, the PHIL-D2-MOD Vector (previously used for the expression of HIV-1 *nef* and *tat* sequences) was used. The recombinant protein is expressed under the control of the inducible alcohol oxidase (AOX1) promoter and the c-terminus of the protein is elongated by a Histidine affinity tail that will facilitate the purification.

<u>10.1 CONSTRUCTION OF THE INTEGRATIVE VECTOR pRIT 14908</u>

To construct **pRIT 14908** , the SIV *nef* gene was amplified by PCR from the pLX5N/SIV-NEF plasmid with primers SNEF1 and SNEF2.

PRIMER SNEF1: 5' ATCGTCC**ATG.**GGTGGAGCTATTTT 3'
NcoI

PRIMER SNEF2: 5' CGGCTACTAGTGCGAGTTTCCTT 3'
SpeI

The SIV *nef* DNA region amplified starts at nucleotide 9077 and terminates at nucleotide 9865 (Aids Research and Human Retroviruses, 6:1221-1231,1990).

An NcoI restriction site (with carries the ATG codon of the *nef* gene) was introduced at the 5' end of the PCR fragment while a SpeI site was introduced at the 3' end.
The PCR fragment obtained and the integrative PHIL-D2-MOD vector were both restricted by NcoI and SpeI. Since one NcoI restriction site is present on the SIV *nef* amplified sequence (at position 9286), two fragments of respectively ±200bp and ± 600bp were obtained, purified on agarose gel and ligated to PHIL-D2-MOD vector.
The resulting recombinant plasmid received, after verification of the *nef* amplified region by automated sequencing, the pRIT 14908 denomination.

10.2 TRANSFORMATION OF PICHIA PASTORIS STRAIN GS115(his4).

To obtain *Pichia pastoris* strain expressing SIV *nef*-His, strain GS115 was transformed with a linear NotI fragment carrying only the expression cassette and the HIS4 gene (Fig.11).
This linear NotI DNA fragment ,with homologies at both ends with AOX1 resident *P.pastoris* gene, favors recombination at the AOX1 locus.
Multicopy integrant clones were selected by quantitative dot blot analysis .
One transformant showing the best production level for the recombinant protein was selected and received the **Y1772** denomination.

Strain **Y1772** produces the recombinant **SIV Nef-His** protein, a **272 amino acids** protein which would be composed of:
°Myristic acid
°A methionine, created by the use of NcoI cloning site of PHIL-D2-MOD vector .

°262 amino acids (aa) of Nef protein (starting at aa 2 and extending to aa 263, see Figure 12)
°A threonine and a serine created by the cloning procedure (cloning at SpeI site of PHIL-D2-MOD vector (Fig.11).
°One glycine and six histidines.

Nucleic and Protein sequences are shown on figure 12.

10.3 CHARACTERIZATION OF THE EXPRESSED PRODUCT OF STRAIN Y1772.

Expression level

After 16 hours induction in medium containing 1% methanol as carbon source, abundance of the recombinant Nef-His protein, was estimated at **10%** of total protein (Fig.13 , lanes 3-4).

Solubility

Induced cultures of recombinant strain Y1772 producing the Nef-His protein were centrifuged. Cell pellets were resuspended in breaking buffer, disrupted with 0.5mm glass beads and the cell extracts were centrifuged. The proteins contained in the insoluble pellet (P) and in the soluble supernatant (S) were compared on a Coomassie Blue stained SDS-PAGE10%.
As shown in figure 13, the majority of the recombinant protein from strain Y1772 (lanes 3-4) is associated with the insoluble fraction.

Strain Y1772 which presents a satisfactory recombinant protein expression level is used for the production and purification of SIV Nef-His protein.

Example 11: EXPRESSION OF GP120 IN CHO

A stable CHO-K1 cell line which produces a recombinant gP120 glycoprotein has been established. Recombinant gP120 glycoprotein is a recombinant truncated form of the gP120 envelope protein of HIV-1 isolate W61D. The protein is excreted into the cell culture medium, from which it is subsequently purified.

Construction of gp120 transfection plasmid pRIT13968

The envelope DNA coding sequence (including the 5`exon of tat and rev) of HIV-1 isolate W61D was obtained (Dr. Tersmette, CCB, Amsterdam) as a genomic gp160 envelope containing plasmid W61D (Nco-XhoI). The plasmid was designated pRIT13965.

In order to construct a gp120 expression cassette a stop codon had to be inserted at the amino acid glu 515 codon of the gp160 encoding sequence in pRIT13965 using a primer oligonucleotide sequence (DIR 131) and PCR technology. Primer DIR 131 contains three stop codons (in all open reading frames) and a SalI restriction site.

The complete gp120 envelope sequence was then reconstituted from the N-terminal BamH1-DraI fragment (170 bp) of a gp160 plasmid subclone pW61d env (pRIT13966) derived from pRIT13965, and the DraI-SalI fragment (510 bp) generated by PCR from pRIT13965. Both fragments were gel purified and ligated together into the E.coli plasmid pUC18, cut first by SalI (klenow treated), and then by BamH1. This resulted in plasmid pRIT13967. The gene sequence of the XmaI-SalI fragment (1580 bp) containing the gp120 coding cassette was sequenced and found to be identical to the predicted sequence. Plasmid RIT13967 was ligated into the CHO GS-expression vector pEE14 (Celltech Ltd., UK) by cutting first with BclI (klenow treated) and then by XmaI. The resulting plasmid was designated pRIT13968.

Preparation of Master Cell Bank

The gp120-construct (pRIT13968) was transfected into CHO cells by the classical $CaPO_4$-precipitation/glycerol shock procedure. Two days later the CHOK1 cells were subjected to selective growth medium (GMEM + methionine sulfoximine (MSX) 25 µM + Glutamate + asparagine + 10% Foetal calf serum). Three chosen

transfectant clones were further amplified in 175m² flasks and few cell vials were stored at -80°C. C-env 23,9 was selected for further expansion.

A small prebank of cells was prepared and 20 ampoules were frozen. For preparation of the prebank and the MCB, cells were grown in GMEM culture medium, supplemented with 7.5 % fetal calf serum and containing 50 μM MSX. These cell cultures were tested for sterility and mycoplasma and proved to be negative.

The Master Cell Bank CHOK1 env 23.9 (at passage 12) was prepared using cells derived from the premaster cell bank. Briefly, two ampoules of the premaster seed were seeded in medium supplemented with 7.5% dialysed foetal bovine serum. The cells were distributed in four culture flasks and cultured at 37°C. After cell attachment the culture medium was changed with fresh medium supplemented with 50 μM MSX. At confluence, cells were collected by trypsination and subcultured with a 1/8 split ratio in T-flasks - roller bottle - cell factory units. Cells were collected from cell factory units by trypsination and centrifugation. The cell pellet was resuspended in culture medium supplemented with DMSO as cryogenic preservative. Ampoules were prelabelled, autoclaved and heat-sealed (250 vials). They were checked for leaks and stored overnight at -70°C before storage in liquid nitrogen.

Cell Culture And Production Of Crude Harvest

Two vials from a master cell bank are thawed rapidly. Cells are pooled and inoculated in two T-flasks at 37° ± 1°C with an appropriate culture medium supplemented with 7.5 % dialysed foetal bovine (FBS) serum. When reaching confluence (passage 13), cells are collected by trypsinisation, pooled and expanded in 10 T-flasks as above. Confluent cells (passage 14) are trypsinised and expanded serially in 2 cell factory units (each 6000 cm²; passage 15), then in 10 cell factories (passage 16). The growth culture medium is supplemented with 7.5 % dialysed foetal bovine (FBS) serum and 1% MSX. When cells reach confluence, the growth culture medium is discarded and replaced by "production medium" containing only 1 % dialysed foetal bovine serum and no MSX. Supernatant is collected every two

days (48 hrs-interval) for up to 32 days. The harvested culture fluids are clarified immediately through a 1.2-0.22 μm filter unit and kept at -20°C before purification.

Example 12: PURIFICATION OF HIV GP 120 (W61D CHO) FROM CELL CULTURE FLUID

All purification steps are performed in a cold room at 2-8°C. pH of buffers are adjusted at this temperature and are filtered on 0.2 μm filter. They are tested for pyrogen content (LAL assay). Optical density at 280 nm, pH and conductivity of column eluates are continuously monitored.

(i) Clarified Culture Fluid

The harvested clarified cell culture fluid (CCF) is filter-sterilized and Tris buffer, pH 8.0 is added to 30 mM final concentration. CCF is stored frozen at -20°C until purification.

(ii) Hydrophobic Interaction Chromatography

After thawing, ammonium sulphate is added to the clarified culture fluid up to 1 M. The solution is passed overnight on a TSK/TOYOPEARL-BUTYL 650 M (TOSOHAAS) column, equilibrated in 30 mM Tris buffer- pH 8.0 - 1 M ammonium sulphate. Under these conditions, the antigen binds to the gel matrix. The column is washed with a decreasing stepwise ammonium sulphate gradient. The antigen is eluted at 30 mM Tris buffer- pH 8.0 - 0.25 M ammonium sulphate.

(iii) Anion-exchange Chromatography

After reducing the conductivity of the solution between 5 and 6 mS/cm, the gP120 pool of fractions is loaded onto a Q-sepharose Fast Flow (Pharmacia) column, equilibrated in Tris-saline buffer - pH 8.0. The column is operated on a negative mode, i.e. gP120 does not bind to the gel, while most of the impurities are retained.

(iv) Concentration and diafiltration by ultrafiltration

In order to increase the protein concentration, the gP120 pool is loaded on a FILTRON membrane "Omega Screen Channel", with a 50 kDa cut-off. At the end of the concentration, the buffer is exchanged by diafiltration with 5 mM phosphate

buffer containing $CaCl_2$ 0.3 mM, pH 7.0. If further processing is not performed immediately, the gP120 pool is stored frozen at -20°C. After thawing the solution is filtered onto a 0.2 µM membrane in order to remove insoluble materiel.

(v) Chromatography on hydroxyapatite

The gP120 UF pool is loaded onto a macro-Prep Ceramic Hydroxyapatite, type II (Biorad) column equilibrated in 5 mM phosphate buffer + $CaCl_2$ 0.3 mM, pH 7.0. The column is washed with the same buffer. The antigen passes through the column and impurities bind to the column.

(vi) Cation exchange chromatography

The gP120 pool is loaded on a CM/TOYOPEARL-650 S (TOSOHAAS) column equilibrated in acetate buffer 20 mM, pH 5.0. The column is washed with the same buffer, then acetate 20 mM, pH 5.0 and NaCl 10 mM. The antigen is then eluted by the same buffer containing 80 mM NaCl.

(vii) Ultrafiltration

In order to augment the virus clearance capacity of the purification process, an additional ultrafiltration step is carried out. The gP120 pool is subjected to ultrafiltration onto a FILTRON membrane "Omega Screen Channel", cut-off 150 kDa. This pore-size membrane does not retain the antigen. After the process, the diluted antigen is concentrated on the same type of membrane (Filtron) but with a cut-off of 50 kDa.

(viii) Size exclusion Gel Chromatography

The gP120 pool is applied to a SUPERDEX 200 (PHARMACIA) column in order to exchange the buffer and to eliminate residual contaminants. The column is eluted with phosphate buffer saline (PBS).

(ix) Sterile filtration and storage

Fractions are sterilized by filtration on a 0.2 µM PVDF membrane (Millipore). After sterile filtration, the purified bulk is stored frozen at -20°C up to formulation. The purification scheme is summarized by the flow sheet below.

⇒ Level of purity of the purified bulk estimated by SDS-PAGE analysis (Silver staining / Coomassie Blue / Western Blotting) is ≥ 95%.

⇒ Production yield is around 2.5 mg /L CCF (according to Lowry assay) - Global purification yield is around 25% (according to Elisa assay)

⇒ Purified material is stable 1 week at 37°C (according to WB analysis)

Purification of gp120 from culture fluid

Mark √ indicate steps that are critical for virus removal.

CLARIFIED CULTURE FLUID
↓
HYDROPHOBIC INTERACTION CHROMATOGRAPHY
(BUTYL -TOYOPEARL 650 M)
↓
ANION EXCHANGE CHROMATOGRAPHY √
(NEGATIVE MODE)
(Q-SEPHAROSE)
↓
50 KD ULTRAFILTRATION
(CONCENTRATION AND BUFFER EXCHANGE)
↓
(STORAGE -20°C)
↓
HYDROXYAPATITE CHROMATOGRAPHY
(NEGATIVE MODE)
(MACROPREP CERAMIC HYDROXYAPATITE II)
↓
CATION EXCHANGE CHROMATOGRAPHY
(CM-TOYOPEARL 650 S)
↓
150 KD ULTRAFILTRATION √
(OMEGA MEMBRANES / FILTRON)
↓

50 KD ULTRAFILTRATION
(CONCENTRATION)
↓

SIZE EXCLUSION CHROMATOGRAPHY √
(SUPERDEX 200)
STERILE FILTRATION
↓

PURIFIED BULK
STORAGE -20°C

Example 13: VACCINE PREPARATION

A vaccine prepared in accordance with the invention comprises the expression products of one or more DNA recombinants encoding an antigen. Furthermore, the formulations comprise a mixture of 3 de -O-acylated monophosphoryl lipid A 3D-MPL and QS21 in an oil/water emulsion or an oligonucleotide containing unmethylated CpG dinucleotide motifs and aluminium hydroxide as carrier.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota.

Experiments performed at Smith Kline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral immunity and a T_{H1} type of cellular immunity.

QS21: is a saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it induces both antigen-specific lymphoproliferation and CTLs to several antigens.
Experiments performed at Smith Kline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and T_{H1} type cellular immune responses.

The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprises 5% squalene, 5%

tocopherol, 2% Tween 80 and has an average particle size of 180 nm (see WO 95/17210).

Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to 3D-MPL/QS21 further increases their immunostimulant properties.

Preparation of the oil/water emulsion (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Preparation of oil in water formulation.

Antigens (100 µg gp120, 20 µg NefTat, and 20 µg SIV Nef, alone or in combination) were diluted in 10 fold concentrated PBS pH 6.8 and H_2O before consecutive addition of the oil in water emulsion, 3D-MPL (50µg), QS21 (50µg) and 1 µg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (250µl for a dose of 500µl).

All incubations were carried out at room temperature with agitation.

CpG oligonucleotide (CpG) is a synthetic unmethylated oligonucleotide containing one or several CpG sequence motifs. CpG is a very potent inducer of T_{H1} type immunity compared to the oil in water formulation that induces mainly a mixed T_{H1}/T_{H2} response. CpG induces lower level of antibodies than the oil in water formulation and a good cell mediated immune response. CpG is expected to induce lower local reactogenicity.

Preparation of CpG oligonucleotide solution: CpG dry powder is dissolved in H_2O to give a solution of 5 mg/ml CpG.

Preparation of CpG formulation.

The 3 antigens were dialyzed against NaCl 150 mM to eliminate the phosphate ions that inhibit the adsorption of gp120 on aluminium hydroxide.

The antigens diluted in H_2O (100 µg gp120, 20 µg NefTat and 20 µg SIV Nef) were incubated with the CpG solution (500 µg CpG) for 30 min before adsorption on $Al(OH)_3$ to favor a potential interaction between the His tail of NefTat and Nef antigens and the oligonucleotide (stronger immunostimulatory effect of CpG described when bound to the antigen compared to free CpG). Then were consecutively added at 5 min interval $Al(OH)_3$ (500 µg), 10 fold concentrated NaCl and 1 µg/ml thiomersal as preservative.

All incubations were carried out at room temperature with agitation.

Example 14: IMMUNIZATION AND SHIV CHALLENGE EXPERIMENT IN RHESUS MONKEYS.

First Study

Groups of 4 rhesus monkeys were immunized intramuscularly at 0, 1 and 3 months with the following vaccine compositions:

Group 1:	Adjuvant 2	+ gp120		
Group 2:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 3:	Adjuvant 2		+ NefTat*	+ SIV Nef
Group 4	Adjuvant 6	+ gp120	+ NefTat	+ SIV Nef
Group 5	Adjuvant 2		+ NefTat	+ SIV Nef
Group 6	Adjuvant 2			

Adjuvant 2 comprises squalene/tocopherol/Tween 80/3D-MPL/QS21 and Adjuvant 6 comprises alum and CpG.

Tat* represents mutated Tat, in which Lys41→Ala and in RGD motif Arg78→Lys and Asp80→Glu (Virology 235: 48-64, 1997).

One month after the last immunization all animals were challenged with a pathogenic SHIV (strain 89.6p). From the week of challenge (wk16) blood samples were taken periodically at the indicated time points to determine the % of CD4-positive cells among peripheral blood mononuclear cells by FACS analysis (Figure 14) and the concentration of RNA viral genomes in the plasma by bDNA assay (Figure 15).

Results

All animals become infected after challenge with $SHIV_{89.6p}$.

CD4-positive cells decline after challenge in all animals of groups 1, 3, 5 and 6 except one animal in each of groups 1 and 6 (control group). All animals in group 2 exhibit a slight decrease in CD4-positive cells and recover to baseline levels over time. A similartrend is observed in group 4 animals (Figure 14).

Virus load data are almost the inverse of CD4 data. Virus load declines below the level of detection in ¾ group 2 animals (and in the one control animal that maintains its CD4-positive cells), and the fourth animal shows only marginal virus load. Most of the other animals maintain a high or intermediate virus load (Figure 15).

Surprisingly, anti-Tat and anti-Nef antibody titres measured by ELISA were 2 to 3-fold higher in Group 3 (with mutated Tat) than in Group 5 (the equivalent Group with non-mutated Tat) throughout the course of the study.

At week 68 (56 weeks post challenge) all animals from the groups that had received the full antigen combination (groups 2 and 4) were still alive, while most of the animals in the other groupshad to be euthanized due to AIDS-like symptoms. The surviving animals per group were:

Group 1:	2/4
Group 2:	4/4
Group 3:	0/4
Group 4	4/4
Group 5	0/4
Group 6	1/4

Conclusions

The combination of gp120 and NefTat (in the presence of SIV Nef) prevents the loss of CD4-positive cells, reduces the virus load in animals infected with pathogenic $SHIV_{89.6p}$, and delays or prevents the development of AIDS-like disease symptoms, while gp120 or NefTat/SIV Nef alone do not protect from the pathologic consequences of the SHIV challenge.

The adjuvant 2 which is an oil in water emulsion comprising squalene, tocopherol and Tween 80, together with 3D-MPL and QS21 seems to have a stronger effect on the study endpoints than the alum / CpG adjuvant.

Second study

A second rhesus monkey SHIV challenge study was conducted to confirm the efficacy of the candidate vaccine gp120/NefTat + adjuvant and to compare different Tat-based antigens. The study was conducted by a different laboratory.

The design of the study was as follows.

Groups of 6 rhesus monkeys were immunized at 0, 4 and 12 weeks with injections i.m. and challenged at week 16 with a standard dose of pathogenic $SHIV_{89.6p.}$

Group 1 is the repeat of Group 2 in the first study.

Group 1:	Adjuvant 2	+ gp120	+ NefTat	+ SIV Nef
Group 2:	Adjuvant 2	+ gp120	+ Tat (oxidised)	
Group 3:	Adjuvant 2	+ gp120	+ Tat (reduced)	
Group 4	Adjuvant 2			

The follow-up/endpoints were again % CD4-positive cells, virus load by RT-PCR, morbidity and mortality

Results

All animals except one in group 2 become infected after challenge with $SHIV_{89.6p}$.

CD4-positive cells decline significantly after challenge in all animals of control group 4 and group 3, and in all but one animals of group 2. Only one animal in group 1 shows a marked decrease in CD4-positive cells. Unlike the animals from the first study, the monkeys in the second experiment display a stabilisation of CD4-positive cells at different levels one month after virus challenge (Figure 16). The stabilisation is generally lower than the initial % of CD4-positive cells, but will never lead to a complete loss of the cells. This may be indicative of a lower susceptibility to SHIV-induced disease in the monkey population that was used for the second study. Nonetheless, a beneficial effect of the gp120/NefTat/SIV Nef vaccine and the two gp120/Tat vaccines is demonstrable. The number of animals with a % of CD4-positive cells above 20 is 5 for the vaccinated animals, while none of the control animals from the adjuvant group remains above that level.

Analysis of RNA plasma virus loads confirms the relatively low susceptibility of the study animals (Figure 17). Only 2 of the 6 control animals maintain a high virus load, while the virus disappears from the plasma in the other animals. Thus, a vaccine effect is difficult to demonstrate for the virus load parameter.

Conclusions

Analysis of CD4-positive cells indicates that the vaccine gp120/NefTat + adjuvant (in the presence of SIV Nef) prevents the drop of CD4-positive cells in most vaccinated

animals This is a confirmation of the result obtained in the first SHIV study. Due to the lack of susceptibility of the study animals, the virus load parameter could not be used to demonstrate a vaccine effect. Taken together, the combination of gp120 and Tat and Nef HIV antigens provides protection against the pathologic consequences of HIV infection, as evidenced in a SHIV model.

The Tat alone antigens in combination with gp120 also provide some protection from the decline of CD4-positive cells. The effect is less pronounced than with the gp120/NefTat/SIV Nef antigen combination, but it demonstrates that gp120 and Tat are able to mediate some protective efficacy against SHIV-induced disease manifestations.

The second SHIV challenge study was performed with rhesus monkeys from a source completely unrelated to the source of animals from the first study. Both parameters, % of CD4-positive cells and plasma virus load, suggest that the animals in the second study were less susceptible to SHIV-induced disease, and that there was considerably greater variability among the animals. Nonetheless, a beneficial effect on the maintenance of CD4-positive cells of the gp120/NefTat/SIV Nef vaccine was seen with the experimental vaccine containing gp120/NefTat and SIV Nef. This indicates that the vaccine effect was not only repeated in a separate study, but furthermore demonstrated in an unrelated monkey population.

CLAIMS

1. Use of a) an HIV Tat protein or polynucleotide; or
b) an HIV Nef protein or polynucleotide; or
c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide (Nef-Tat);
and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine for the prophylactic or therapeutic immunisation of humans against HIV.

2. Use as claimed in claim 1 wherein the Tat, Nef or Nef-Tat act in synergy with gp120 in the treatment or prevention of HIV.

3. Use as claimed in claim 1 or claim 2 wherein the vaccine in use reduces the HIV viral load in HIV infected humans.

4. Use as claimed in claims 1 or 2 wherein the vaccine in use results in a maintenance of CD4+ levels over those levels found in the absence of vaccination with HIV Tat, Nef or Nef-Tat and HIV gp120.

5. Use as claimed in any one of claims 1 – 4 wherein the vaccine further comprises an antigen selected from the group consisting of: gag, rev, vif, vpr, vpu.

6. Use as claimed in any one of claims 1 – 5 wherein the Tat protein is a mutated protein.

7. Use as claimed in any one of claims 1 – 6 wherein the Tat, Nef or Nef-Tat protein is reduced.

8. Use as claimed in any one of claims 1 – 7 wherein the Tat, Nef or Nef-Tat protein is carbamidomethylated.

9. Use as claimed in any one of claims 1 – 6 wherein the Tat, Nef or Nef-Tat protein is oxidised.

10. Use as claimed in any one of claims 1 – 9 which additionally comprises an adjuvant.

11. Use as claimed in claim 10 wherein the adjuvant is a TH1 inducing adjuvant.

12. Use as claimed in claim 10 or claim 11 wherein the adjuvant comprises monophosphoryl lipid A or a derivative thereof such as 3-de-O-acylated monophsphoryl lipid A.

13. Use as claimed in any one of claims 10 – 12 additionally comprising a saponin adjuvant.

14. Use as claimed in any one of claims 10 – 13 additionally comprising an oil in water emulsion.

15. Use as claimed in claim 10 or claim 11 wherein the adjuvant comprises CpG motif-containing oligonucleotides.

16. Use as claimed in claim 15 further comprising an aluminium salt.

17. Use of a) an HIV Tat protein or polynucleotide; or

b) an HIV Nef protein or polynucleotide; or

c) an HIV Tat protein or polynucleotide linked to an HIV Nef protein or polynucleotide;

and an HIV gp120 protein or polynucleotide in the manufacture of a vaccine suitable for a prime-boost delivery for the prophylactic or therapeutic immunisation of humans against HIV.

18. A method of immunising a human against HIV by administering to the human a vaccine comprising HIV Tat or HIV Nef or HIV NefTat in combination with HIV gp120 proteins or polynucleotides encoding them.

19. A vaccine composition for human use which vaccine composition comprises HIV Tat or HIV Nef or HIV Nef-Tat in combination with HIV gp120 proteins or polynucleotides encoding them.

FIGURE 1

The DNA and amino acid sequences of Nef-His; Tat-His; Nef-Tat-His fusion and mutated Tat is illustrated.

Pichia-expressed constructs (plain constructs)

⇒ *Nef - HIS*

DNA sequence (Seq. ID. No. 8)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGC
CACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 9)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ *Tat - HIS*

DNA sequence (Seq. ID. No. 10)

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAA
ACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTC
ATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAA

TCCCGAGGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence (Seq. ID. No. 11)

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRR
PPQGSQTHQVSLSKQPTSQSRGDPTGPKETSGHHHHHH.

⇒ *Nef - Tat - HIS*

DNA sequence (Seq. ID. No. 12)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGA
ATGAGACGAGCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAA
AAACATGGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGG
CTAGAAGCACAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTA
AGACCAATGACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGG
GGACTGGAAGGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATC
TACCACACACAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTC
AGATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAG
GTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCAT
GGAATGGATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCA
TTTCATCACGTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAG
CCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCT
TGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA
AAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCT
CAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGA
GGGGACCCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

Protein sequence(Seq. ID. No. 13)

MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAW
LEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWI
YHTQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLH
GMDDPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTA
CTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSR
GDPTGPKETSGHHHHHH.

E.coli-expressed constructs (fusion constructs)

⇒ *LipoD-Nef-HIS*

DNA sequence (Seq. ID. No. 14)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

*ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGT**TGT
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCTTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCACCATCACCAT
TAA

Protein sequence of the processed lipidated ProtD-Nef-HIS protein (Seq. ID. No. 15)

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSGHHHHHH.

⇒ *LipoD-Nef-Tat-HIS*

DNA sequence (Seq. ID. No. 16)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.
The Lipidation Signal Sequence is underlined. After processing, the cysteine coded by the TGT codon, indicated with a star, becomes the amino terminal residue which is then modified by covalently bound fatty acids.

*

<u>ATGGATCCAAAAACTTTAGCCCTTTCTTTATTAGCAGCTGGCGTACTAGCAGGTTGT</u>
AGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAAATCATTATT
GCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCTAAAGCACTT
GCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACTAAGGATGGT
CGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAA
TTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAA
GAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGCAAGTGGTCA
AAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGAAAGAATGAGACGAGCTGAGCCA
GCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGAGCAATCACA
AGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCACAAGAGGAG
GAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACTTACAAG
GCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAAGGGCTAATT
CACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCTTT
GGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAA
GGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGATGACCCTGAG
AGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA
GAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTAGACTA
GAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGT
AAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCC
TATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCAT
CAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCGACAGGCCCG
AAGGAAACTAGTGGCCACCATCACCATCACCATTAA

<u>Protein sequence of the processed lipidated ProtD-NEF-TAT-HIS protein (Seq. ID. No. 17)</u>

(Amino-acids corresponding to Prot D fusion partner are in bold)

CSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMTKD
GRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGGKW
SKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEAQE
EEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQG
YFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDP
EREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCY
CKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTG
PKETSGHHHHHH.

⇒ *ProtD-Nef -HIS*

DNA sequence (Seq. ID. No. 18)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGGCCACCATCAC
CATCACCATTAA

Protein sequence (Seq. ID. No. 19)

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLK
EIQSLEMTENFETMGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDL
EKHGAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSH
FLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRYPLTFGW
CYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRFDSRLAFH
HVARELHPEYFKNCTSGHHHHHH.

⇒ *ProtD-Nef -Tat-HIS*

DNA sequence (Seq. ID. No. 20)

Nucleotides corresponding to the Prot D Fusion Partner are in bold.

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATCAGACAAA
ATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGCATACGTTAGAATCT
AAAGCACTTGCGTTTGCACAACAGGCTGATTATTTAGAGCAAGATTTAGCAATGACT
AAGGATGGTCGTTTAGTGGTTATTCACGATCACTTTTTAGATGGCTTGACTGATGTT
GCGAAAAAATTCCCACATCGTCATCGTAAAGATGGCCGTTACTATGTCATCGACTTT
ACCTTAAAAGAAATTCAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGGTGGC
AAGTGGTCAAAAAGTAGTGTGGTTGGATGGCCTACTGTAAGGGAAAGAATGAGACGA
GCTGAGCCAGCAGCAGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACATGGA
GCAATCACAAGTAGCAATACAGCAGCTACCAATGCTGCTTGTGCCTGGCTAGAAGCA
CAAGAGGAGGAGGAGGTGGGTTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATG
ACTTACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAAAGGGGGGACTGGAA
GGGCTAATTCACTCCCAACGAAGACAAGATATCCTTGATCTGTGGATCTACCACACA
CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCA
CTGACCTTTGGATGGTGCTACAAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAG
GCCAATAAAGGAGAGAACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGGAT
GACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAGCCGCCTAGCATTTCATCAC
GTGGCCCGAGAGCTGCATCCGGAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGAT
CCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAACTGCTTGTACCAAT
TGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTA
GGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGT
CAGACTCATCAAGTTTCTCTATCAAAGCAACCCACCTCCCAATCCCGAGGGGACCCG
ACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATCACCATTAA

<u>*Protein sequence (Seq. ID. No. 21)*</u>

(Amino-acids corresponding to Prot D fusion partner are in bold)

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYLEQDLAMT
KDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMGG
KWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKHGAITSSNTAATNAACAWLEA
QEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHT
QGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMD
DPEREVLEWRFDSRLAFHHVARELHPEYFKNCTSEPVDPRLEPWKHPGSQPKTACTN
CYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDP
TGPKETSGHHHHHH.

<u>*⇒ Tat-MUTANT-HIS*</u>

<u>*DNA sequence (Seq. ID. No. 22)*</u>

ATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATC 40
CAGGAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTG 80
TAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACA 120
GCTGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGAC 160
AGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAGT 200
TTCTCTATCAAAGCAACCCACCTCCCAATCCAAAGGGGAG 240
CCGACAGGCCCGAAGGAAACTAGTGGCCACCATCACCATC 280
ACCATTAA 288

Protein sequence(Seq. ID. No. 23)

Mutated amino-acids in Tat sequences are in bold.

MEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFIT 40
AALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSKGE 80
PTGPKETSGHHHHHH. 95

⇒*Nef-Tat-Mutant-HIS*

DNA sequence(Seq. ID. No. 24)

ATGGGTGGCAAGTGGTCAAAAAGTAGTGTGGTTGGATGGC 40
CTACTGTAAGGGAAAGAATGAGACGAGCTGAGCCAGCAGC 80
AGATGGGGTGGGAGCAGCATCTCGAGACCTGGAAAAACAT 120
GGAGCAATCACAAGTAGCAATACAGCAGCTACCAATGCTG 160
CTTGTGCCTGGCTAGAAGCACAAGAGGAGGAGGAGGTGGG 200
TTTTCCAGTCACACCTCAGGTACCTTTAAGACCAATGACT 240
TACAAGGCAGCTGTAGATCTTAGCCACTTTTTAAAAGAAA 280
AGGGGGGACTGGAAGGGCTAATTCACTCCCAACGAAGACA 320
AGATATCCTTGATCTGTGGATCTACCACACACAAGGCTAC 360
TTCCCTGATTGGCAGAACTACACACCAGGGCCAGGGGTCA 400
GATATCCACTGACCTTTGGATGGTGCTACAAGCTAGTACC 440
AGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAG 480
AACACCAGCTTGTTACACCCTGTGAGCCTGCATGGAATGG 520
ATGACCCTGAGAGAGAAGTGTTAGAGTGGAGGTTTGACAG 560
CCGCCTAGCATTTCATCACGTGGCCCGAGAGCTGCATCCG 600
GAGTACTTCAAGAACTGCACTAGTGAGCCAGTAGATCCTA 640
GACTAGAGCCCTGGAAGCATCCAGGAAGTCAGCCTAAAAC 680
TGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCAT 720
TGCCAAGTTTGTTTCATAACAGCTGCCTTAGGCATCTCCT 760
ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCA 800
AGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAACCC 840
ACCTCCCAATCCAAAGGGGAGCCGACAGGCCCGAAGGAAA 880
CTAGTGGCCACCATCACCATCACCATTAA 909

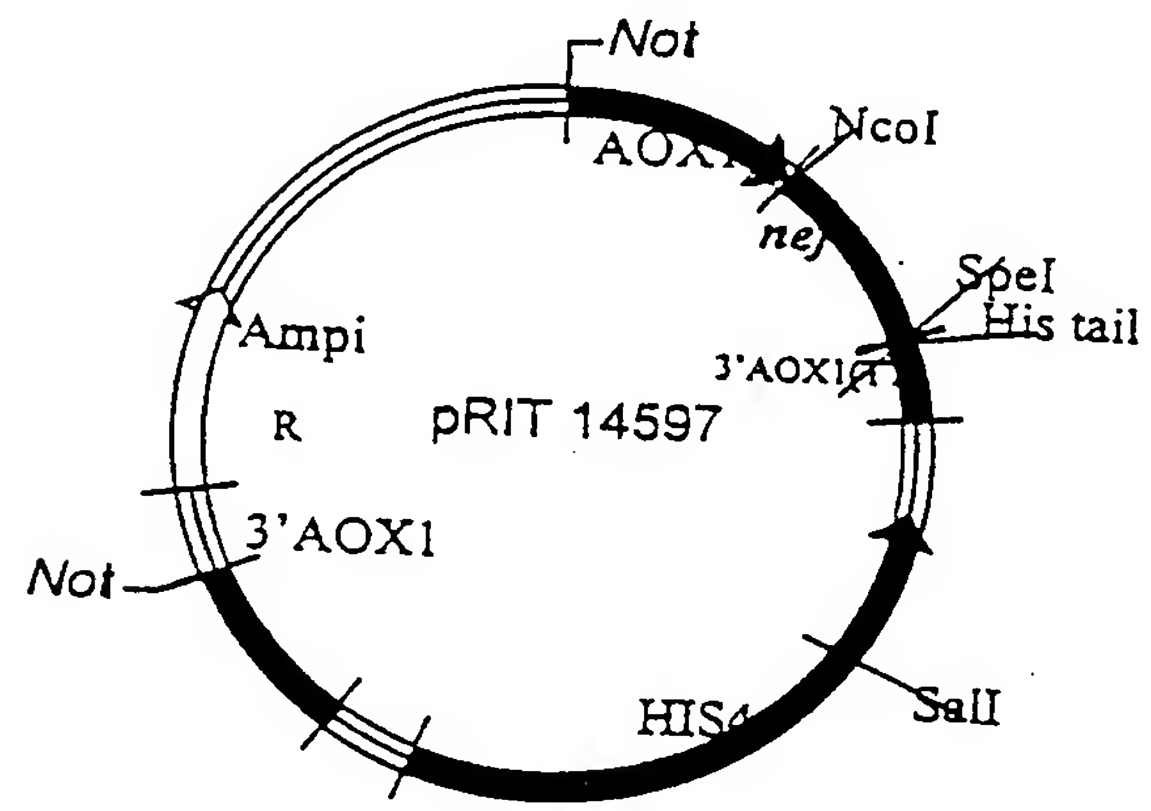
Protein sequence (Seq. ID. No. 25)

Mutated amino-acids in Tat sequence are in bold.

```
MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLEKH  40
GAITSSNTAATNAACAWLEAQEEEEVGFPVTPQVPLRPMT  80
YKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGY 120
FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE 160
NTSLLHPVSLHGMDDPEREVLEWRFDSRLAFHHVARELHP 200
EYFKNCTSEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFH 240
CQVCFITAALGISYGRKKRRQRRRPPQGSQTHQVSLSKQP 280
TSQSKGEPTGPKETSGHHHHHH.                  302
```

Figure 2

Map of pRIT14597 integrative vector



MCS POLYLINKER: *nef* gene inserted between NcoI and SpeI sites.

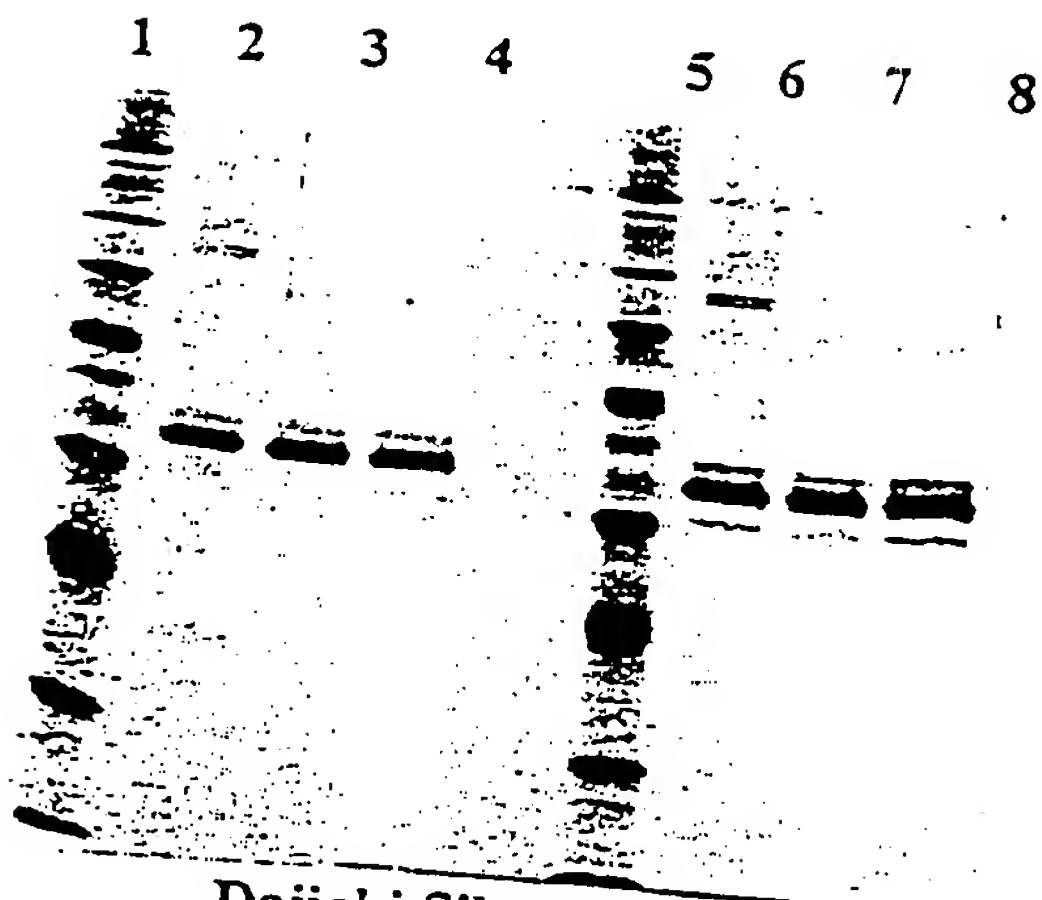
Asu II *Nco* I *Spe* I *Eco* RI

TTCGAA.ACC.ATGGCCGCGGACTAGTGGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGGAATTC

Thr .Ser . Gly. His . His . His . His . His . His

The amino acid sequence of Figure 2 relates to Seq. ID no. 27 and the nucleic acid sequence of Figure 3 relates to Seq. ID. No. 26.

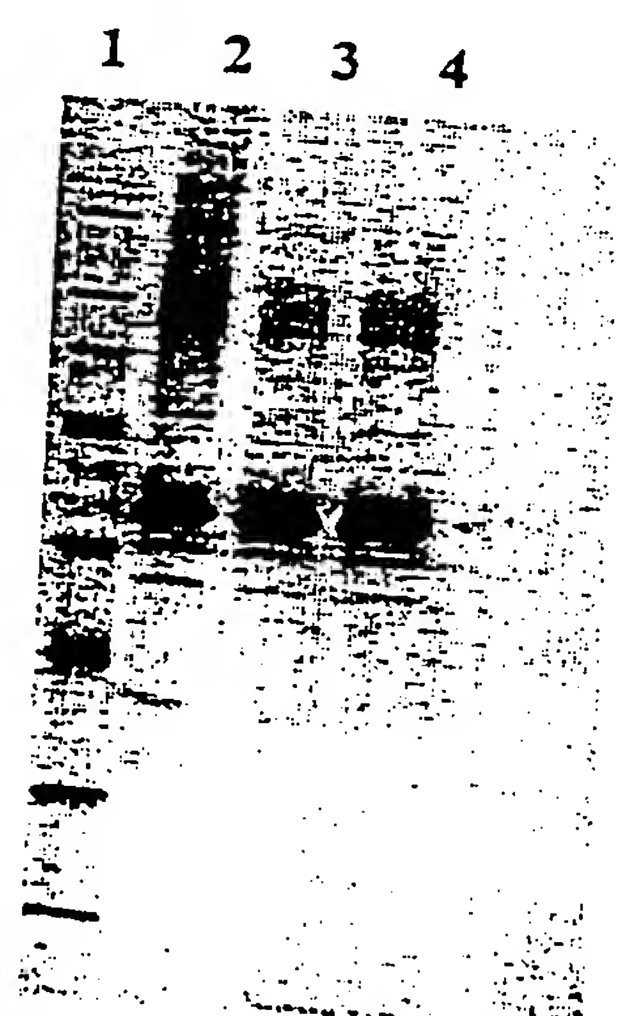
Figure 3 : SDS-PAGE: Nef-Tat-his fusion protein



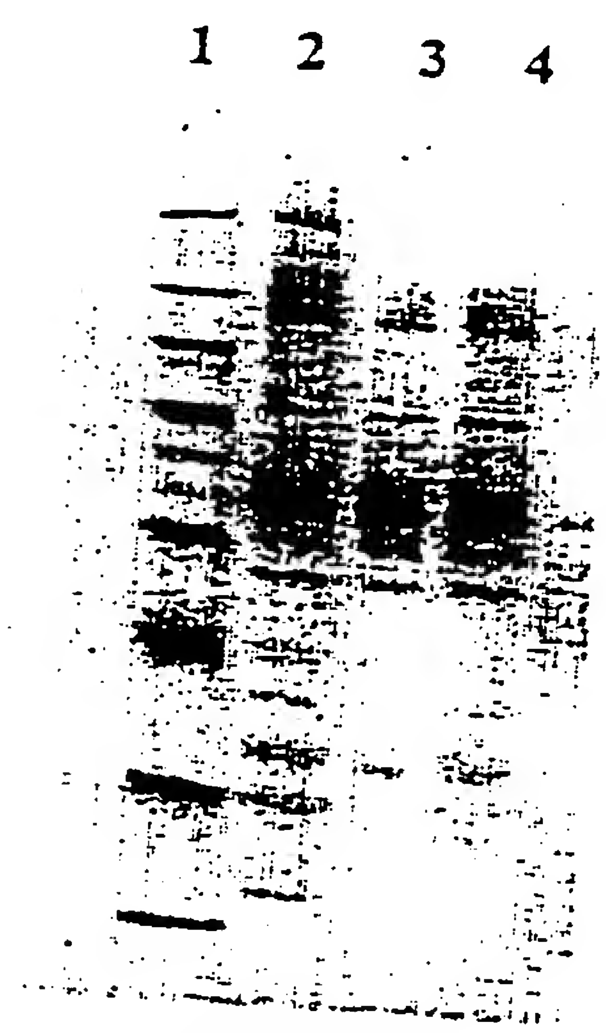
Daiichi Silver Staining

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
2: TNH/23 SP eluate (250 ng)
3: TNH/23 Purified bulk (250 ng)
4: TNH/22 Purified bulk (250 ng)

5: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa
6: TNH/23 SP eluate (400 ng)
7: TNH/23 Purified bulk (400 ng)
8: TNH/22 Purified bulk (400 ng)

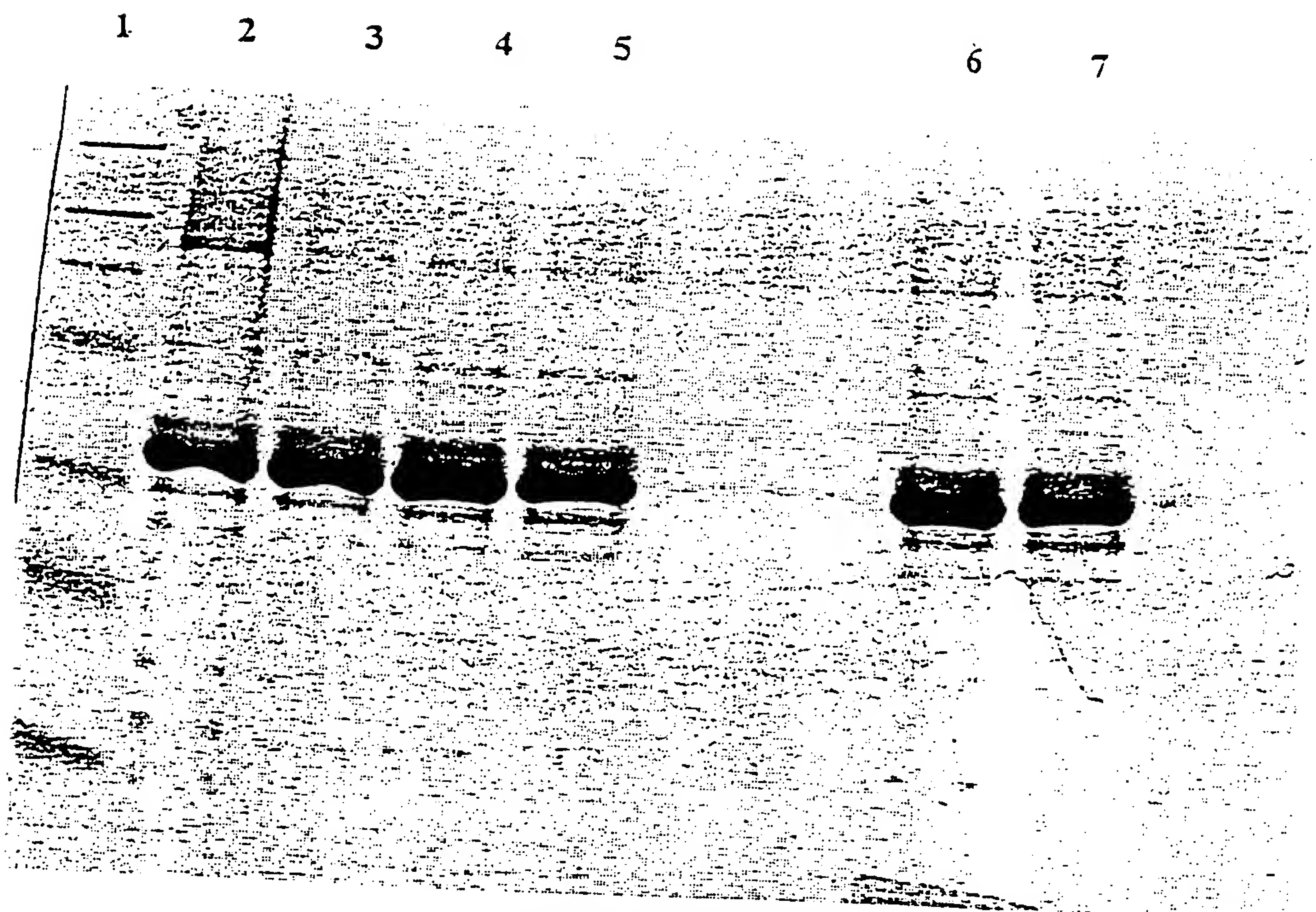


Blot α Nef-Tat (LAS 97340)



Blot Tat2

Figure 4 : SDS-PAGE: Nef-Tat-his fusion protein

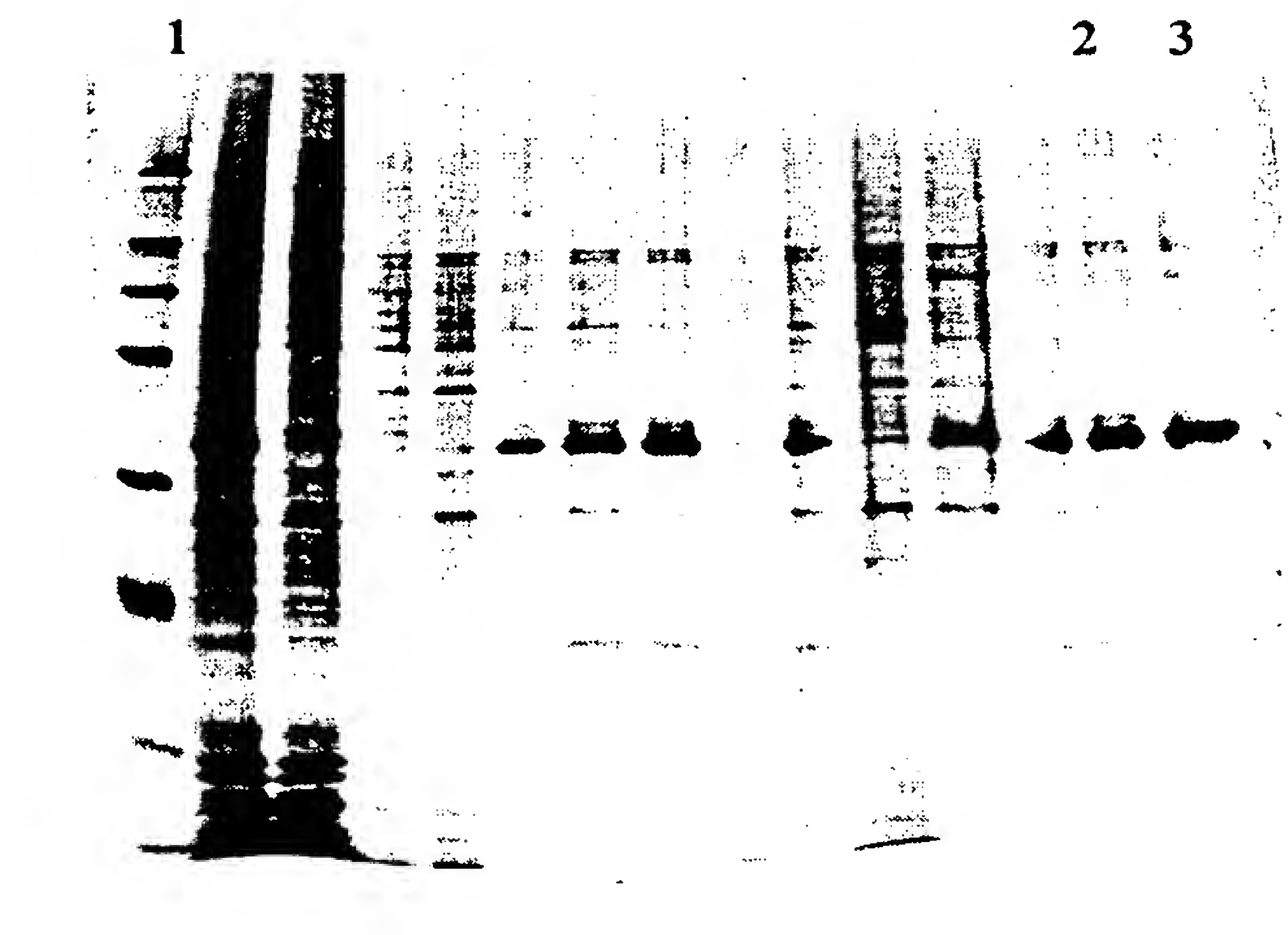


Coomassie blue G250

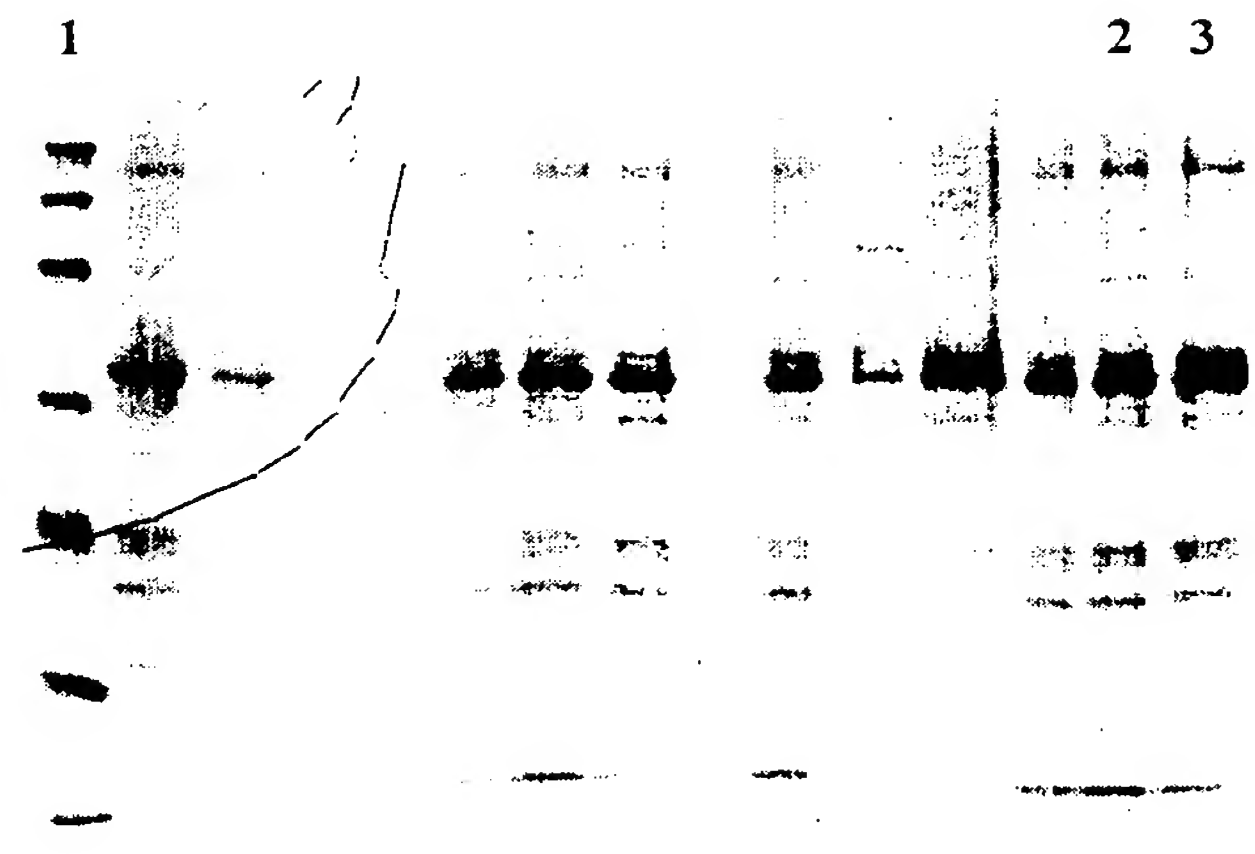
1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: TNH/23 SP eluate (4 µg)
3: TNH/23 Superdex200 eluate (4 µg)
4: TNH/23 Purified bulk (4 µg)
5: TNH/22 Purified bulk (4 µg)

6: TNH/23 Purified bulk (4 µg) / non reducing conditions
7: TNH/22 Purified bulk (4 µg) / non reducing conditions

Figure 6: SDS-PAGE ANALYSIS – reducing conditions (14% polyacrylamide precasted gels - Novex) See example 5



Silver staining



Western blot α Tat

1: MW (175/83/62/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk
3: Purified bulk

Figure 7 (relating to Example 6): SDS-PAGE ANALYSIS:
(4-20% polyacrylamide precasted gels - Novex)

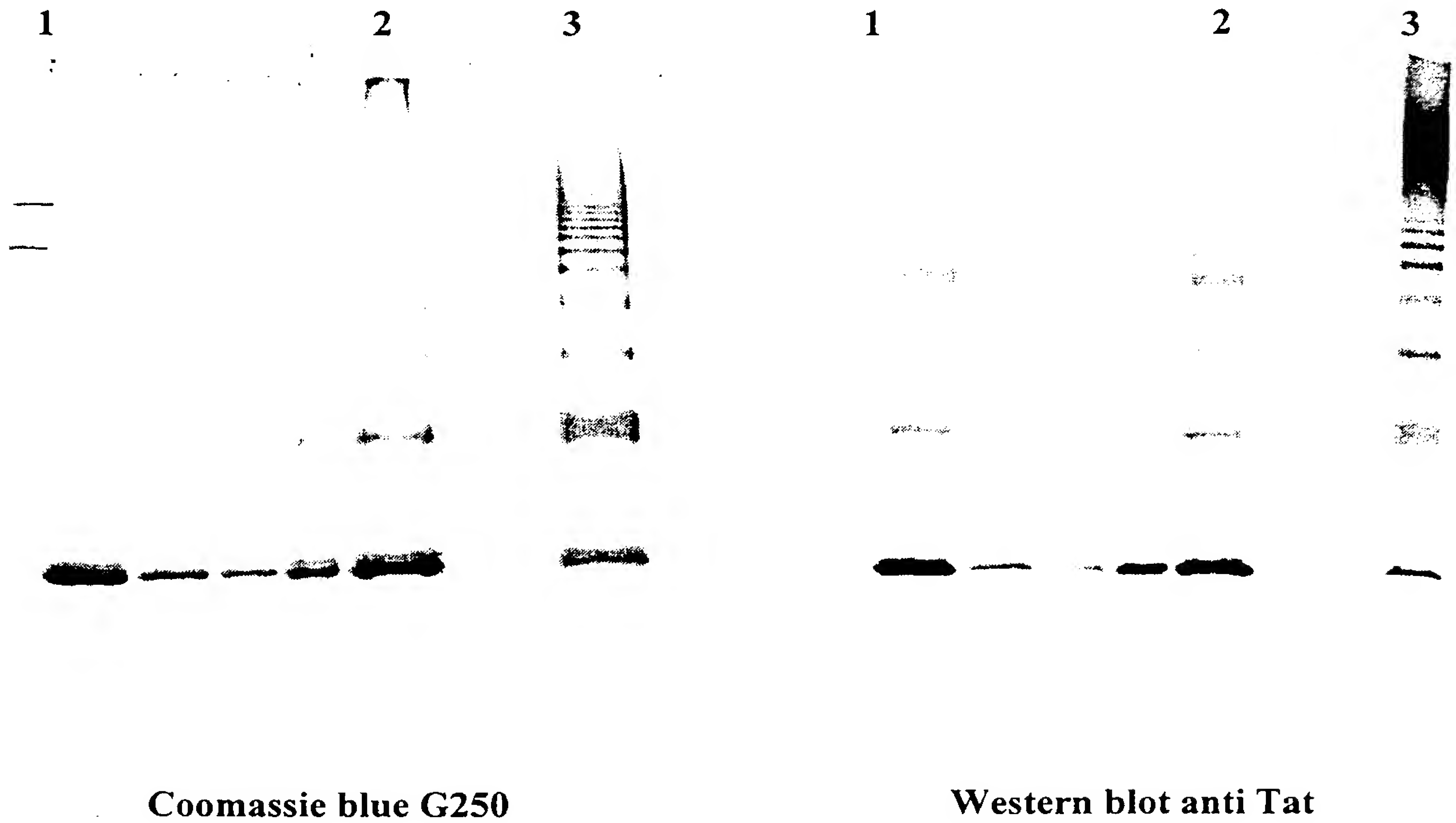
1 2 3 4 5 6 7 1 2 3 4 5 6 7

Coomassie blue G250 **Western blot anti Tat**

1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (reducing conditions)
4: Purified bulk (reducing conditions)

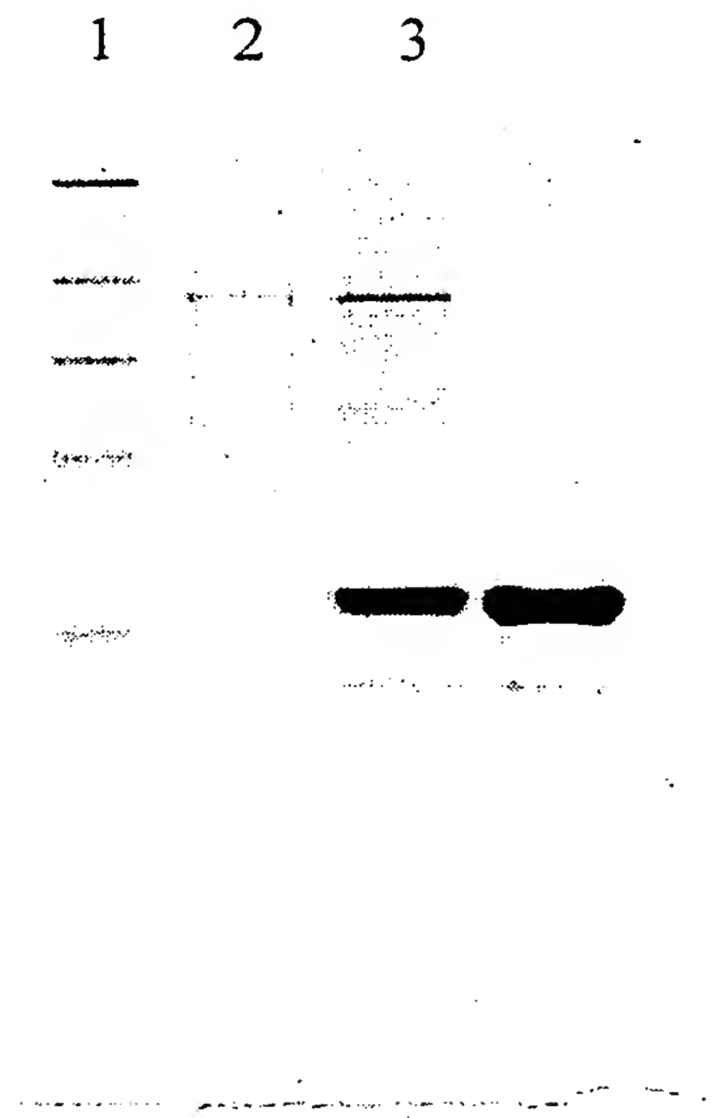
5: Purified bulk (non reducing conditions)
6: Purified bulk (non reducing conditions)
7: Purified bulk (non reducing conditions)

Figure 8 (relating to Example 7): SDS-PAGE ANALYSIS:
(4-20% polyacrylamide precasted gels - Novex)

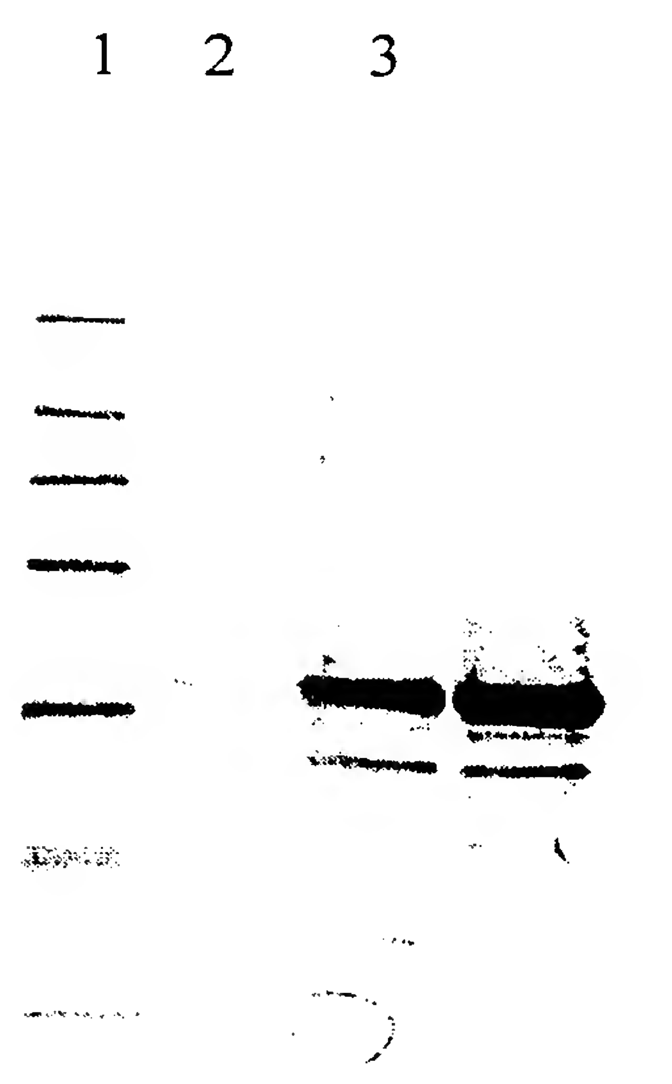


1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk (reducing conditions)
3: Purified bulk (non reducing conditions)

FIGURE 9: SDS-PAGE ANALYSIS - REDUCING CONDITIONS
(14% polyacrylamide precasted gels - Novex) see Example 8

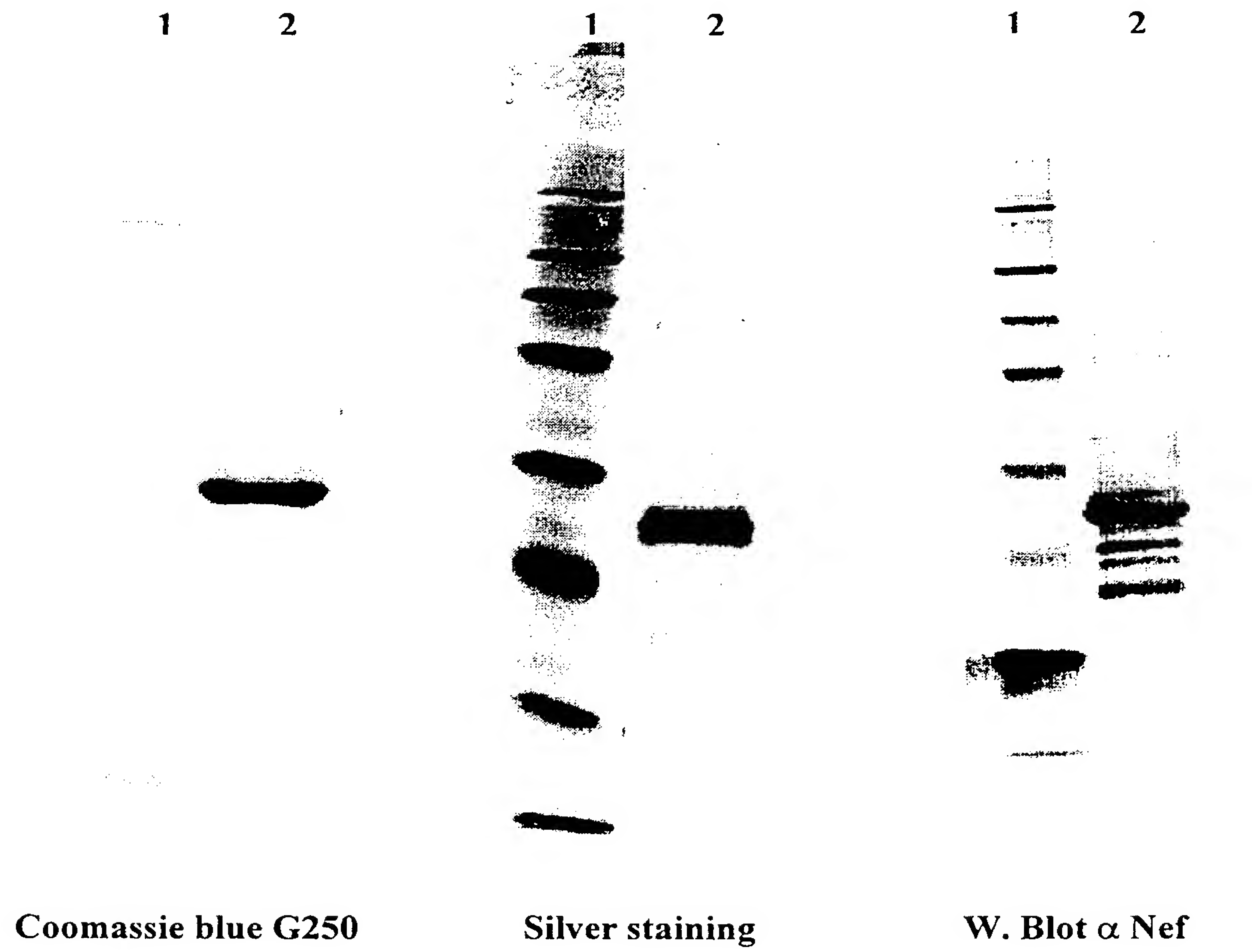


Coomassie blue R250



Silver staining

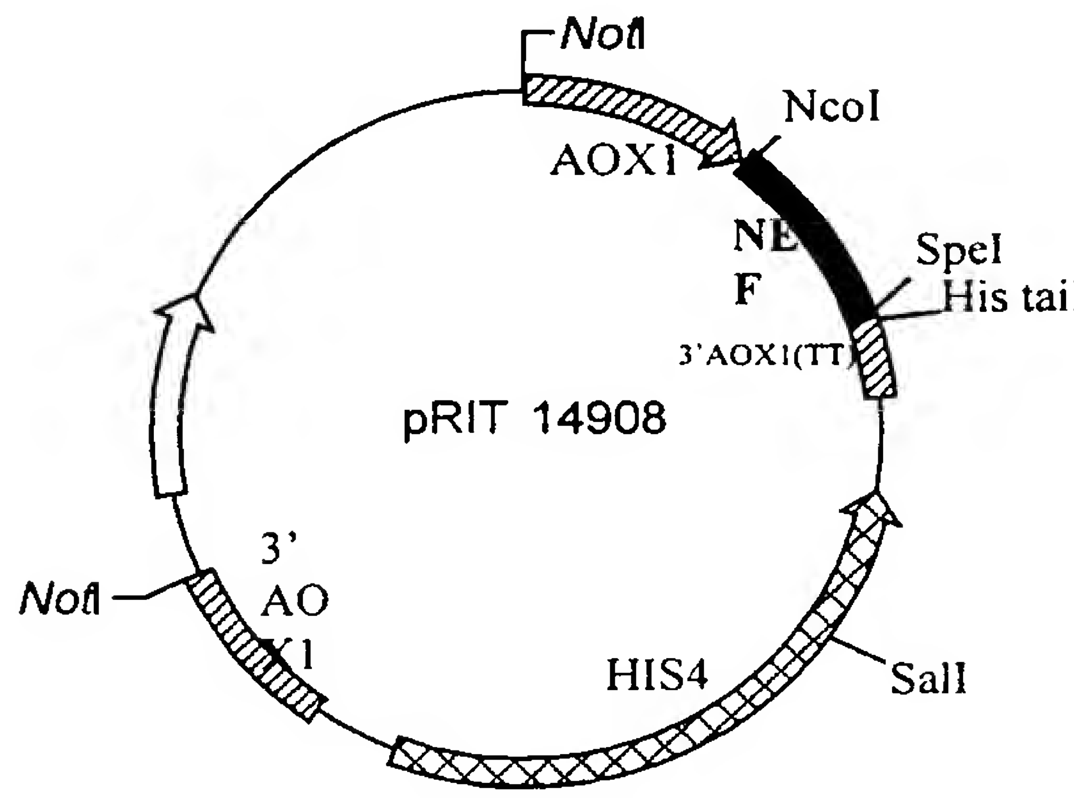
Figure 10: SDS-PAGE ANALYSIS – REDUCING CONDITIONS
(14% polyacrylamide precasted gels - Novex) See Example 9



1: MW (175/83/62,5/47,5/32,5/25/16,5/6,5 kDa)
2: Purified bulk

Figure 11

Map of pRIT14908 integrative vector



MCS POLYLINKER : NEF gene inserted between NcoI and SpeI sites.

Asu II *Nco* I *Spe* I *Eco* RI

TTCGAA.A CC.**ATG** GCCGCGG ACTAGT .GGC.CAC.CAT.CAC.CAT.CAC.CAT.TAA.CGC GAATTC

Thr .Ser . Gly. His . His . His . His . His . His .

Figure 12

Sequences of Pichia-expressed SIV-NEF-His protein

DNA SEQUENCE:

```
atgggtggagctatttccatgaggcggtccaggccgtctggagatctgcg   50
acagagactcttgcgggcgcgtggggagacttatggagactcttaggag  100
aggtggaagatggatactcgcaatccccaggaggattagacaagggcttg  150
agctcactctcttgtgagggacagaaatacaatcagggacagtatatgaa  200
tactccatggagaaacccagctgaagagagagaaaaattagcatacagaa  250
aacaaaatatggatgatatagatgaggaagatgatgacttggtaggggta  300
tcagtgaggccaaaagttcccctaagaacaatgagttacaaattggcaat  350
agacatgtctcattttataaaagaaaaggggggactggaagggatttatt  400
acagtgcaagaagacatagaatcttagacatatacttagaaaaggaagaa  450
ggcatcataccagattggcaggattacacctcaggaccaggaattagata  500
cccaaagacatttggctggctatggaaattagtccctgtaaatgtatcag  550
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tcccagtgggatgacccttggggagaggttctagcatggaagtttgatcc  650
aactctggcctacacttatgaggcatatgttagatacccagaagagtttg  700
gaagcaagtcaggcctgtcagaggaagaggttagaagaaggctaaccgca  750
agaggccttcttaacatggctgacaagaaggaaactcgcactagtggcca  800
ccatcaccatcaccattaa.                                819
```

PROTEIN SEQUENCE:

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MGGAISMRRSRPSGDLRQRLLRARGETYGRLLGEVEDGYSQSPGGLDKGL   50
SSLSCEGQKYNQGQYMNTPWRNPAEEREKLAYRKQNMDDIDEEDDDLVGV  100
SVRPKVPLRTMSYKLAIDMSHFIKEKGGLEGIYYSARRHRILDIYLEKEE  150
GIIPDWQDYTSGPGIRYPKTFGWLWKLVPVNVSDEAQEDEEHYLMHPAQT  200
SQWDDPWGEVLAWKFDPTLAYTYEAYVRYPEEFGSKSGLSEEEVRRRLTA  250
RGLLNMADKKETRTSGHHHHHH.                             272
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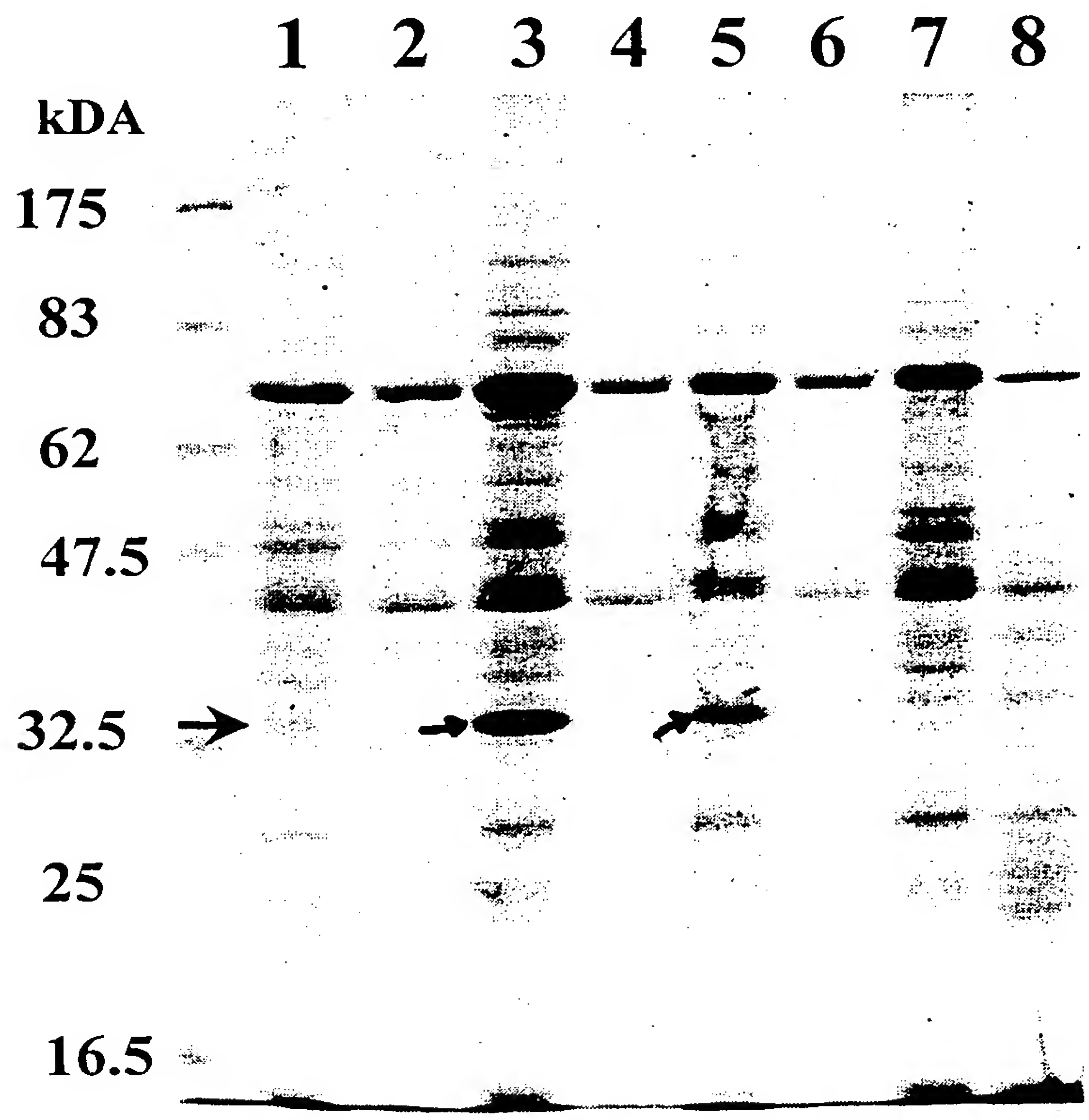
Figure 13

Coomassie Blue Stained SDS-PAGE of recombinant Pichia pastoris SIV/NEF expressing strains



lane 1: P- Y1752 strain
lane 2: S- " "
lane 3: P- Y1772 strain
lane 4: S- " " "
lane 7: P- GS115 strain (negative control)
lane 8: S- " "

Figure 14. Monkey study 1. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

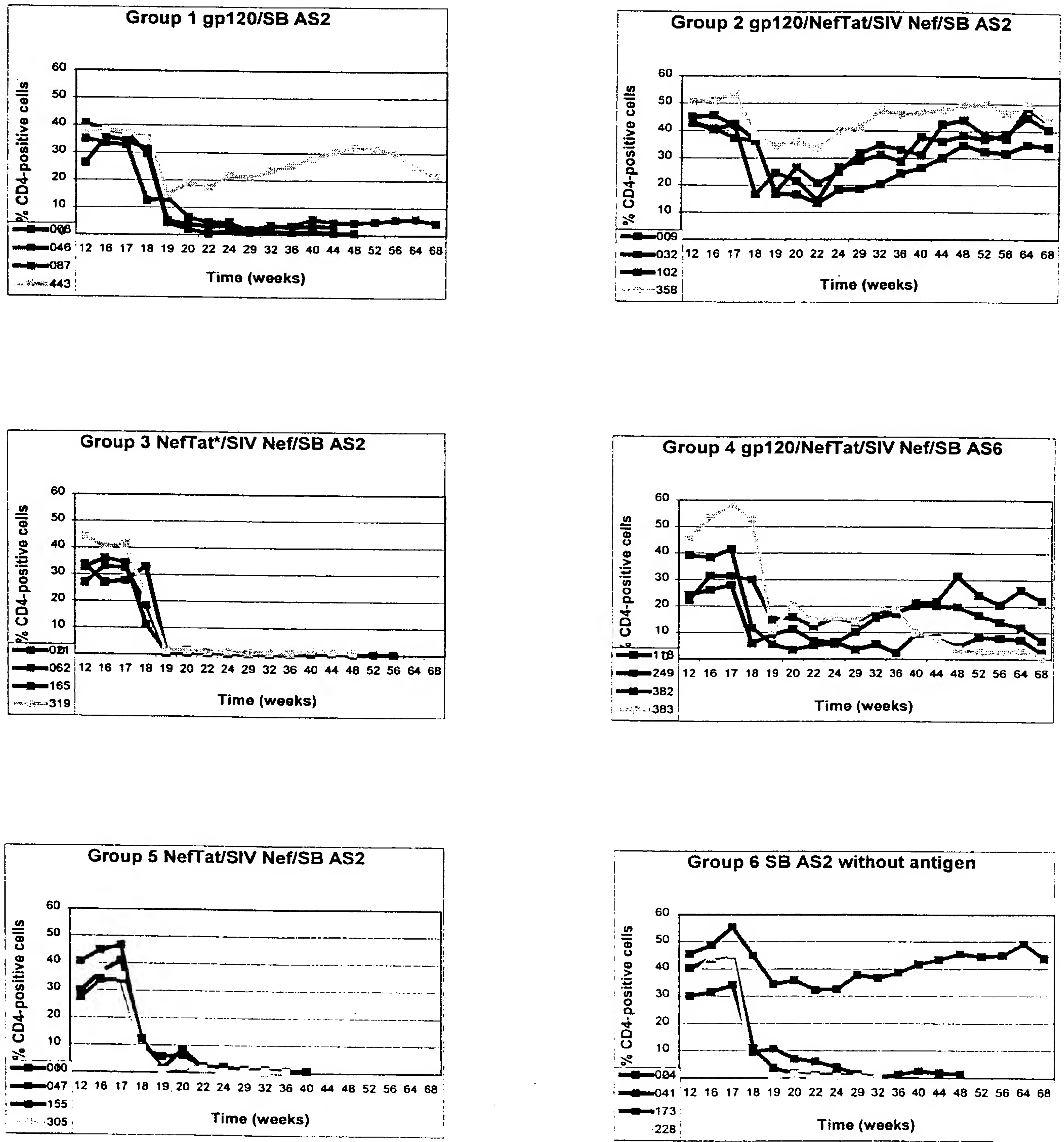


Figure 15. Monkey study 1. Analysis of SHIV plasma virus load after challenge with SHIV

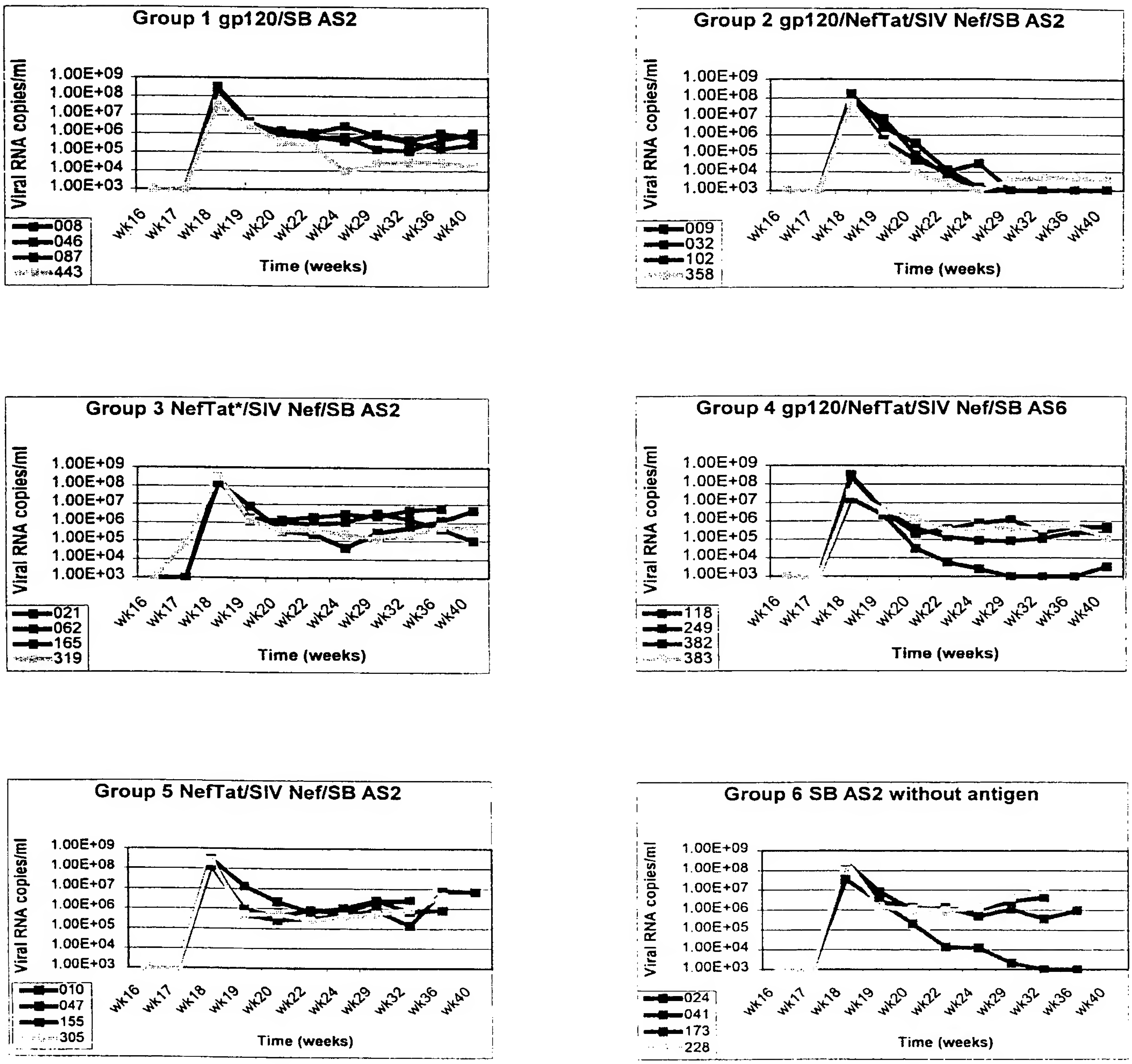


Figure 16. Monkey study 2. Analysis of CD4-positive cells among PBMCs before and after challenge with SHIV

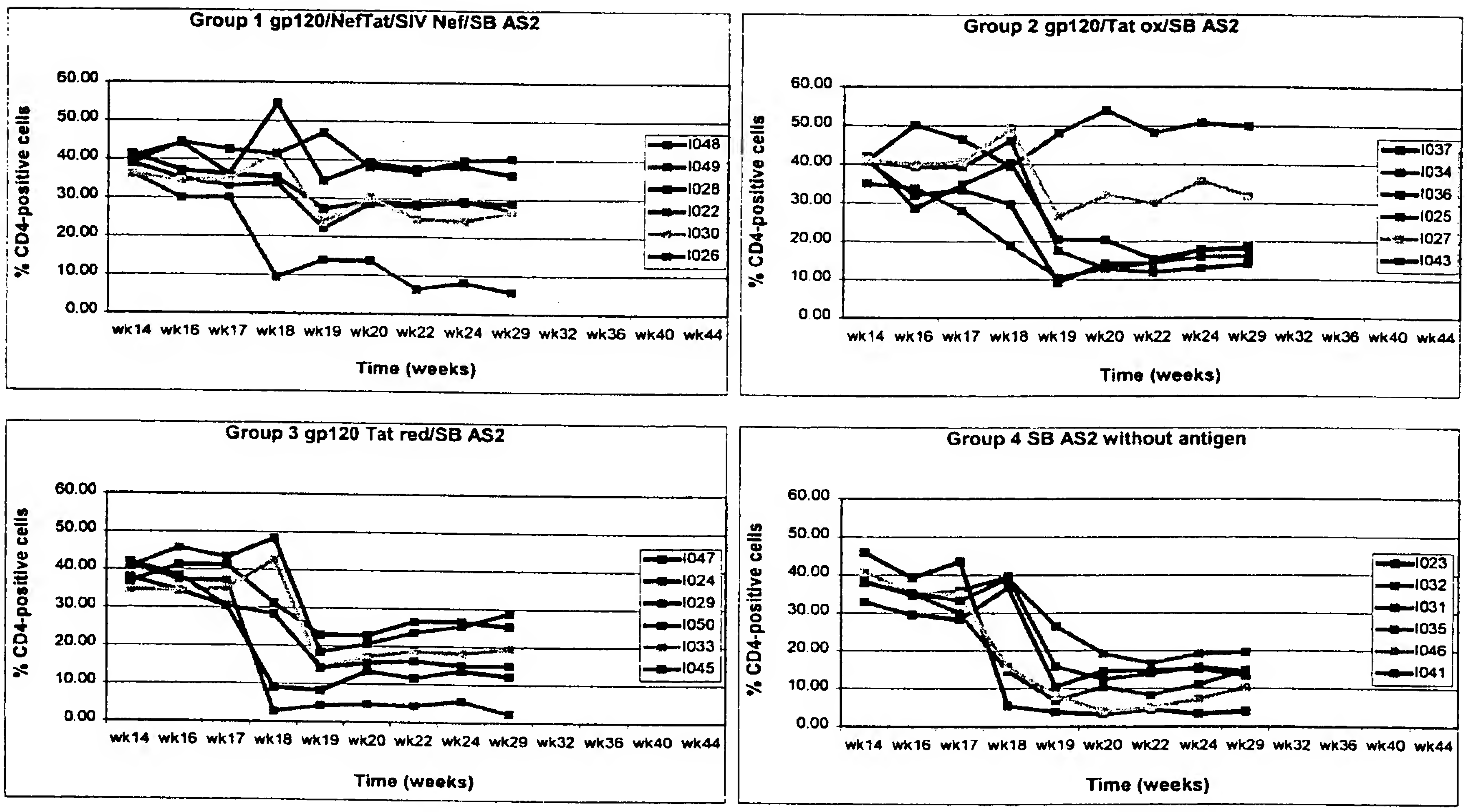
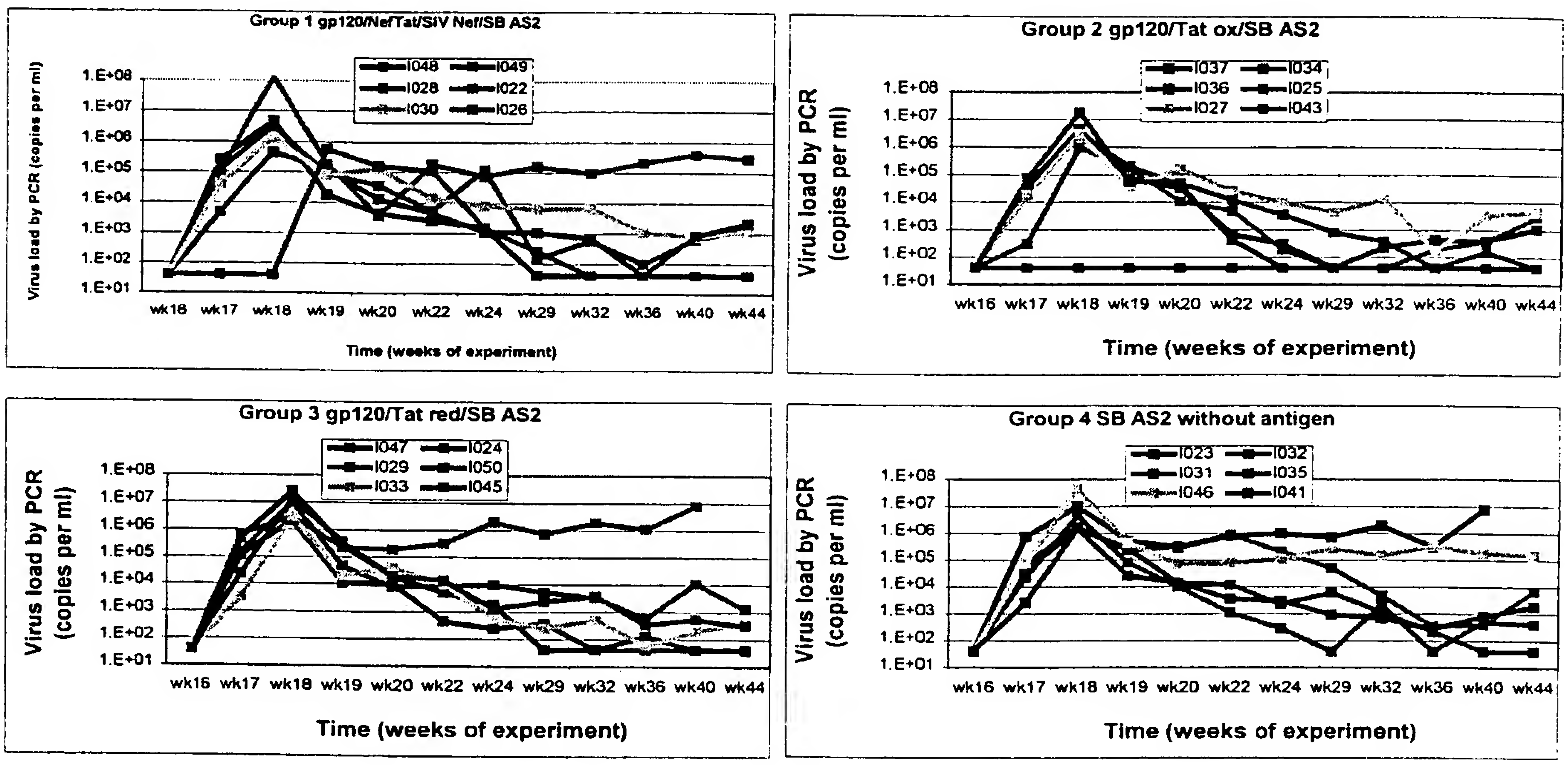


Figure 17. Monkey study 2. Analysis of SHIV plasma virus load after challenge with SHIV



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<120> Novel Use

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<160> 31

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ggagcaatca caagtagcaa tacagcagct accaatgctg cttgtgcctg gctagaagca     180
caagaggagg aggaggtggg ttttccagtc acacctcagg tacctttaag accaatgact     240
tacaaggcag ctgtagatct tagccacttt ttaaaagaaa aggggggact ggaagggcta     300
attcactccc aacgaagaca agatatcctt gatctgtgga tctaccacac acaaggctac     360
ttccctgatt ggcagaacta cacaccaggg ccaggggtca gatatccact gacctttgga     420
tggtgctaca agctagtacc agttgagcca gataaggtag aagaggccaa taaaggagag     480
aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg     540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg     600
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<212> PRT
<213> human

<400> 9
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1               5                   10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
```

```
                100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
            115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Gly His His His His His His
    210                 215
```

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<211> 288
<212> DNA
<213> human

<400> 10

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aaagccttag gcatctccta tggcaggaag aagcggagac agcgacgaag acctcctcaa     180
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc ccgaggggac     240
ccgacaggcc cgaaggaaac tagtggccac catcaccatc accattaa                  288
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<210> 11
<211> 95
<212> PRT
<213> human

<400> 11

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 1               5                  10                  15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
            20                  25                  30
His Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly
        35                  40                  45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
    50                  55                  60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp
65                  70                  75                  80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
                85                  90                  95
```

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<211> 909
<212> DNA
<213> human

<400> 12

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agacgagctg agccagcagc agatggggtg ggagcagcat ctcgagacct ggaaaaacat     120
ggagcaatca caagtagcaa tacagcagct accaatgctg cttgtgcctg gctagaagca     180
caagaggagg aggaggtggg ttttccagtc acacctcagg tacctttaag accaatgact     240
tacaaggcag ctgtagatct tagccacttt ttaaaagaaa aggggggact ggaagggcta     300
attcactccc aacgaagaca agatatcctt gatctgtgga tctaccacac acaaggctac     360
ttccctgatt ggcagaacta cacaccaggg ccaggggtca gatatccact gacctttgga     420
tggtgctaca agctagtacc agttgagcca gataaggtag aagaggccaa taaaggagag     480
aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg     540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg     600
gagtacttca agaactgcac tagtgagcca gtagatccta gactagagcc ctggaagcat     660
```

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ccaggaagtc agcctaaaac tgcttgtacc aattgctatt gtaaaaagtg ttgctttcat      720
tgccaagttt gtttcataac aaaagcctta ggcatctcct atggcaggaa gaagcggaga      780
cagcgacgaa gacctcctca aggcagtcag actcatcaag tttctctatc aaagcaaccc      840
acctcccaat cccgagggga cccgacaggc ccgaaggaaa ctagtggcca ccatcaccat      900
caccattaa                                                              909

        <210> 13
        <211> 302
        <212> PRT
        <213> human

        <400> 13
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1               5                   10                  15
Arg Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala
            20                  25                  30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
        35                  40                  45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
    50                  55                  60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65                  70                  75                  80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
                85                  90                  95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
            100                 105                 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr
        115                 120                 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
    130                 135                 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145                 150                 155                 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
                165                 170                 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
            180                 185                 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
        195                 200                 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
    210                 215                 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225                 230                 235                 240
Cys Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg
                245                 250                 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
            260                 265                 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro
        275                 280                 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
    290                 295                 300

        <210> 14
        <211> 1029
        <212> DNA
        <213> human

        <400> 14
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agccattcat caaatatggc gaatacccaa atgaaatcag acaaaatcat tattgctcac      120
cgtggtgcta gcggttattt accagagcat acgttagaat ctaaagcact tgcttttgca      180
caacaggctg attatttaga gcaagattta gcaatgacta aggatggtcg tttagtggtt      240
attcacgatc actttttaga tggcttgact gatgttgcga aaaaattccc acatcgtcat      300
cgtaaagatg gccgttacta tgtcatcgac tttaccttaa aagaaattca aagtttagaa      360
atgacagaaa actttgaaac catgggtggc aagtggtcaa aaagtagtgt ggttggatgg      420
```

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cctactgtaa gggaaagaat gagacgagct gagccagcag cagatggggt gggagcagca     480
tctcgagacc tggaaaaaca tggagcaatc acaagtagca atacagcagc taccaatgct     540
gcttgtgcct ggctagaagc acaagaggag gaggaggtgg gttttccagt cacacctcag     600
gtacctttaa gaccaatgac ttacaaggca gctgtagatc ttagccactt tttaaaagaa     660
aaggggggac tggaagggct aattcactcc caacgaagac aagatatcct tgatctgtgg     720
atctaccaca cacaaggcta cttccctgat tggcagaact acacaccagg gccaggggtc     780
agatatccac tgacctttgg atggtgctac aagctagtac cagttgagcc agataaggta     840
gaagaggcca ataaaggaga gaacaccagc ttgttacacc ctgtgagcct gcatggaatg     900
gatgaccctg agagagaagt gttagagtgg aggtttgaca gccgcctagc atttcatcac     960
gtggcccgag agctgcatcc ggagtacttc aagaactgca ctagtggcca ccatcaccat    1020
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      <210> 15
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      <212> PRT
      <213> human

      <400> 15
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1               5                   10                  15
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            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
            100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
        115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
    130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
            260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
        275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly His His
305                 310                 315                 320
His His His His


      <210> 16
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      <212> DNA
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<213> human

<400> 16

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cgtggtgcta gcggttattt accagagcat acgttagaat ctaaagcact tgcgtttgca     180
caacaggctg attatttaga gcaagattta gcaatgacta aggatggtcg tttagtggtt     240
attcacgatc actttttaga tggcttgact gatgttgcga aaaaattccc acatcgtcat     300
cgtaaagatg gccgttacta tgtcatcgac tttaccttaa aagaaattca aagtttagaa     360
atgacagaaa actttgaaac catgggtggc aagtggtcaa aaagtagtgt ggttggatgg     420
cctactgtaa gggaaagaat gagacgagct gagccagcag cagatggggt gggagcagca     480
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gcttgtgcct ggctagaagc acaagaggag gaggaggtgg gttttccagt cacacctcag     600
gtacctttaa gaccaatgac ttacaaggca gctgtagatc ttagccactt tttaaaagaa     660
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tatggcagga agaagcggag acagcgacga agacctcctc aaggcagtca gactcatcaa    1200
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<212> PRT
<213> human

<400> 17

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            20                  25                  30
Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
        35                  40                  45
Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
    50                  55                  60
Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
65                  70                  75                  80
Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
                85                  90                  95
Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met Gly Gly
            100                 105                 110
Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg Glu Arg
        115                 120                 125
Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala Ser Arg
    130                 135                 140
Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala Ala Thr
145                 150                 155                 160
Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu Val Gly
                165                 170                 175
Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr Lys Ala
            180                 185                 190
Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly
        195                 200                 205
Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp Ile Tyr
    210                 215                 220
His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro Gly Pro
225                 230                 235                 240
Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu Val Pro
                245                 250                 255
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Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn Thr Ser
                260                 265                 270
Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu Arg Glu
            275                 280                 285
Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His Val Ala
    290                 295                 300
Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu Pro Val
305                 310                 315                 320
Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro Lys Thr
                325                 330                 335
Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Gln Val
                340                 345                 350
Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg
            355                 360                 365
Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln Val Ser
    370                 375                 380
Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr Gly Pro
385                 390                 395                 400
Lys Glu Thr Ser Gly His His His His His His
                405                 410
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<212> DNA
<213> human

<400> 18

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cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt     180
cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc     240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt     300
caaagtttag aaatgacaga aaactttgaa accatgggtg gcaagtggtc aaaaagtagt     360
gtggttggat ggcctactgt aagggaaaga atgagacgag ctgagccagc agcagatggg     420
gtgggagcag catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca     480
gctaccaatg ctgcttgtgc ctggctagaa gcacaagagg aggaggaggt gggttttcca     540
gtcacacctc aggtaccttt aagaccaatg acttacaagg cagctgtaga tcttagccac     600
tttttaaaag aaaagggggg actggaaggg ctaattcact cccaacgaag acaagatatc     660
cttgatctgt ggatctacca cacacaaggc tacttccctg attggcagaa ctacacacca     720
gggccagggg tcagatatcc actgaccttt ggatggtgct acaagctagt accagttgag     780
ccagataagg tagaagaggc caataaagga gagaacacca gcttgttaca ccctgtgagc     840
ctgcatggaa tggatgaccc tgagagagaa gtgttagagt ggaggtttga cagccgccta     900
gcatttcatc acgtggcccg agagctgcat ccggagtact tcaagaactg cactagtggc     960
caccatcacc atcaccatta a                                                981
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<210> 19
<211> 326
<212> PRT
<213> human

<400> 19

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 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
                20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
            35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
```

100 105 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
115 120 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
130 135 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145 150 155 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
165 170 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
180 185 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
195 200 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
210 215 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225 230 235 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
245 250 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
260 265 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
275 280 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
290 295 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Gly
305 310 315 320
His His His His His His
325

<210> 20
<211> 1242
<212> DNA
<213> human

<400> 20
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attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca 120
cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180
cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300
caaagtttag aaatgacaga aaactttgaa accatgggtg gcaagtggtc aaaaagtagt 360
gtggttggat ggcctactgt aagggaaaga atgagacgag ctgagccagc agcagatggg 420
gtgggagcag catctcgaga cctggaaaaa catggagcaa tcacaagtag caatacagca 480
gctaccaatg ctgcttgtgc ctggctagaa gcacaagagg aggaggaggt gggttttcca 540
gtcacacctc aggtaccttt aagaccaatg acttacaagg cagctgtaga tcttagccac 600
tttttaaaag aaaagggggg actggaaggg ctaattcact cccaacgaag acaagatatc 660
cttgatctgt ggatctacca cacacaaggc tacttccctg attggcagaa ctacacacca 720
gggccagggg tcagatatcc actgaccttt ggatggtgct acaagctagt accagttgag 780
ccagataagg tagaagaggc caataaagga gagaacacca gcttgttaca ccctgtgagc 840
ctgcatggaa tggatgaccc tgagagagaa gtgttagagt ggaggtttga cagccgccta 900
gcatttcatc acgtggcccg agagctgcat ccggagtact tcaagaactg cactagtgag 960
ccagtagatc ctagactaga gccctggaag catccaggaa gtcagcctaa aactgcttgt 1020
accaattgct attgtaaaaa gtgttgcttt cattgccaag tttgtttcat aacaaaagcc 1080
ttaggcatct cctatggcag gaagaagcgg agacagcgac gaagacctcc tcaaggcagt 1140
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ggcccgaagg aaactagtgg ccaccatcac catcaccatt aa 1242

<210> 21
<211> 413
<212> PRT
<213> human

<400> 21

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 1               5                  10                  15
Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30
Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80
Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95
Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110
Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val Arg
        115                 120                 125
Glu Arg Met Arg Arg Ala Glu Pro Ala Ala Asp Gly Val Gly Ala Ala
    130                 135                 140
Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr Ala
145                 150                 155                 160
Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu Glu
                165                 170                 175
Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr Tyr
            180                 185                 190
Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly Leu
        195                 200                 205
Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu Trp
    210                 215                 220
Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr Pro
225                 230                 235                 240
Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys Leu
                245                 250                 255
Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu Asn
            260                 265                 270
Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro Glu
        275                 280                 285
Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His His
    290                 295                 300
Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser Glu
305                 310                 315                 320
Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln Pro
                325                 330                 335
Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys
            340                 345                 350
Gln Val Cys Phe Ile Thr Lys Ala Leu Gly Ile Ser Tyr Gly Arg Lys
        355                 360                 365
Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His Gln
    370                 375                 380
Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Arg Gly Asp Pro Thr
385                 390                 395                 400
Gly Pro Lys Glu Thr Ser Gly His His His His His His
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<210> 22
<211> 288
<212> DNA
<213> human

<400> 22

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gctgccttag gcatctccta tggcaggaag aagcggagac agcgacgaag acctcctcaa    180
ggcagtcaga ctcatcaagt ttctctatca aagcaaccca cctcccaatc caaaggggag    240
ccgacaggcc cgaaggaaac tagtggccac catcaccatc accattaa                 288
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<210> 23
<211> 95
<212> PRT
<213> human

<400> 23
Met Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser
1 5 10 15
Gln Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe
20 25 30
His Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly
35 40 45
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr
50 55 60
His Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu
65 70 75 80
Pro Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
85 90 95

<210> 24
<211> 909
<212> DNA
<213> human

<400> 24
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ggagcaatca caagtagcaa tacagcagct accaatgctg cttgtgcctg gctagaagca 180
caagaggagg aggaggtggg ttttccagtc acacctcagg tacctttaag accaatgact 240
tacaaggcag ctgtagatct tagccacttt ttaaaagaaa aggggggact ggaagggcta 300
attcactccc aacgaagaca agatatcctt gatctgtgga tctaccacac acaaggctac 360
ttccctgatt ggcagaacta cacaccaggg ccaggggtca gatatccact gacctttgga 420
tggtgctaca agctagtacc agttgagcca gataaggtag aagaggccaa taaaggagag 480
aacaccagct tgttacaccc tgtgagcctg catggaatgg atgaccctga gagagaagtg 540
ttagagtgga ggtttgacag ccgcctagca tttcatcacg tggcccgaga gctgcatccg 600
gagtacttca agaactgcac tagtgagcca gtagatccta gactagagcc ctggaagcat 660
ccaggaagtc agcctaaaac tgcttgtacc aattgctatt gtaaaaagtg ttgctttcat 720
tgccaagttt gtttcataac agctgcctta ggcatctcct atggcaggaa gaagcggaga 780
cagcgacgaa gacctcctca aggcagtcag actcatcaag tttctctatc aaagcaaccc 840
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caccattaa 909

<210> 25
<211> 302
<212> PRT
<213> human

<400> 25
Met Gly Gly Lys Trp Ser Lys Ser Ser Val Val Gly Trp Pro Thr Val
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20 25 30
Ala Ser Arg Asp Leu Glu Lys His Gly Ala Ile Thr Ser Ser Asn Thr
35 40 45
Ala Ala Thr Asn Ala Ala Cys Ala Trp Leu Glu Ala Gln Glu Glu Glu
50 55 60
Glu Val Gly Phe Pro Val Thr Pro Gln Val Pro Leu Arg Pro Met Thr
65 70 75 80
Tyr Lys Ala Ala Val Asp Leu Ser His Phe Leu Lys Glu Lys Gly Gly
85 90 95
Leu Glu Gly Leu Ile His Ser Gln Arg Arg Gln Asp Ile Leu Asp Leu
100 105 110
Trp Ile Tyr His Thr Gln Gly Tyr Phe Pro Asp Trp Gln Asn Tyr Thr

115 120 125
Pro Gly Pro Gly Val Arg Tyr Pro Leu Thr Phe Gly Trp Cys Tyr Lys
130 135 140
Leu Val Pro Val Glu Pro Asp Lys Val Glu Glu Ala Asn Lys Gly Glu
145 150 155 160
Asn Thr Ser Leu Leu His Pro Val Ser Leu His Gly Met Asp Asp Pro
165 170 175
Glu Arg Glu Val Leu Glu Trp Arg Phe Asp Ser Arg Leu Ala Phe His
180 185 190
His Val Ala Arg Glu Leu His Pro Glu Tyr Phe Lys Asn Cys Thr Ser
195 200 205
Glu Pro Val Asp Pro Arg Leu Glu Pro Trp Lys His Pro Gly Ser Gln
210 215 220
Pro Lys Thr Ala Cys Thr Asn Cys Tyr Cys Lys Lys Cys Cys Phe His
225 230 235 240
Cys Gln Val Cys Phe Ile Thr Ala Ala Leu Gly Ile Ser Tyr Gly Arg
245 250 255
Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Ser Gln Thr His
260 265 270
Gln Val Ser Leu Ser Lys Gln Pro Thr Ser Gln Ser Lys Gly Glu Pro
275 280 285
Thr Gly Pro Lys Glu Thr Ser Gly His His His His His His
290 295 300

<210> 26
<211> 57
<212> DNA
<213> human

<400> 26
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<210> 27
<211> 9
<212> PRT
<213> human

<400> 27
Thr Ser Gly His His His His His His
1 5

<210> 28
<211> 58
<212> DNA
<213> human

<400> 28
ttcgaaacca tggccgcgga ctagtggcca ccatcaccat caccattaac gcgaattc 58

<210> 29
<211> 9
<212> PRT
<213> human

<400> 29
Thr Ser Gly His His His His His His
1 5

<210> 30
<211> 819
<212> DNA
<213> human

<400> 30

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caatccccag gaggattaga caagggcttg agctcactct cttgtgaggg acagaaatac 180
aatcagggac agtatatgaa tactccatgg agaaacccag ctgaagagag agaaaaatta 240
gcatacagaa aacaaaatat ggatgatata gatgaggaag atgatgactt ggtaggggta 300
tcagtgaggc caaaagttcc cctaagaaca atgagttaca aattggcaat agacatgtct 360
cattttataa aagaaaaggg gggactggaa gggatttatt acagtgcaag aagacataga 420
atcttagaca tatacttaga aaaggaagaa ggcatcatac cagattggca ggattacacc 480
tcaggaccag gaattagata cccaaagaca tttggctggc tatggaaatt agtccctgta 540
aatgtatcag atgaggcaca ggaggatgag gagcattatt taatgcatcc agctcaaact 600
tcccagtggg atgacccttg gggagaggtt ctagcatgga agtttgatcc aactctggcc 660
tacacttatg aggcatatgt tagataccca gaagagtttg gaagcaagtc aggcctgtca 720
gaggaagagg ttagaagaag gctaaccgca agaggccttc ttaacatggc tgacaagaag 780
gaaactcgca ctagtggcca ccatcaccat caccattaa 819

<210> 31
<211> 272
<212> PRT
<213> human

<400> 31
Met Gly Gly Ala Ile Ser Met Arg Arg Ser Arg Pro Ser Gly Asp Leu
1 5 10 15
Arg Gln Arg Leu Leu Arg Ala Arg Gly Glu Thr Tyr Gly Arg Leu Leu
20 25 30
Gly Glu Val Glu Asp Gly Tyr Ser Gln Ser Pro Gly Gly Leu Asp Lys
35 40 45
Gly Leu Ser Ser Leu Ser Cys Glu Gly Gln Lys Tyr Asn Gln Gly Gln
50 55 60
Tyr Met Asn Thr Pro Trp Arg Asn Pro Ala Glu Glu Arg Glu Lys Leu
65 70 75 80
Ala Tyr Arg Lys Gln Asn Met Asp Asp Ile Asp Glu Glu Asp Asp Asp
85 90 95
Leu Val Gly Val Ser Val Arg Pro Lys Val Pro Leu Arg Thr Met Ser
100 105 110
Tyr Lys Leu Ala Ile Asp Met Ser His Phe Ile Lys Glu Lys Gly Gly
115 120 125
Leu Glu Gly Ile Tyr Tyr Ser Ala Arg Arg His Arg Ile Leu Asp Ile
130 135 140
Tyr Leu Glu Lys Glu Glu Gly Ile Ile Pro Asp Trp Gln Asp Tyr Thr
145 150 155 160
Ser Gly Pro Gly Ile Arg Tyr Pro Lys Thr Phe Gly Trp Leu Trp Lys
165 170 175
Leu Val Pro Val Asn Val Ser Asp Glu Ala Gln Glu Asp Glu Glu His
180 185 190
Tyr Leu Met His Pro Ala Gln Thr Ser Gln Trp Asp Asp Pro Trp Gly
195 200 205
Glu Val Leu Ala Trp Lys Phe Asp Pro Thr Leu Ala Tyr Thr Tyr Glu
210 215 220
Ala Tyr Val Arg Tyr Pro Glu Glu Phe Gly Ser Lys Ser Gly Leu Ser
225 230 235 240
Glu Glu Glu Val Arg Arg Arg Leu Thr Ala Arg Gly Leu Leu Asn Met
245 250 255
Ala Asp Lys Lys Glu Thr Arg Thr Ser Gly His His His His His His
260 265 270